Biennial Report 2006–2007

Biozentrum
University of Basel

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BIOZENTRUM
Universität Basel

Biennial Report 2006–2007

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Preface

The turn-over in the Biozentrum faculty as a consequence of retirements continued in 2006/07 and 6 new colleagues were appointed in this period. At present, the Biozentrum comprises 33 research groups with 9 female group leaders.

Molecular Biology, Structural and Dynamic Biophysics, and Bioinformatics remain the core activities of the Biozentrum with the research being centered on three „Focal Areas“:

- Cell Growth & Development
- Infection Biology
- Neurobiology

Additionally, two method-oriented „Core Programs“ have been developed as scientific „glue“. These are:

- Structural Biology and Biophysics
- Systems Biology and Computational Science

Our undergraduate/graduate program in „Molecular Biology“ has been redesigned to correspond to the Bologna system, leading to a Bachelor diploma in 3 years, followed by a fast-track Master thesis/program after an additional 1-1.5 years. The program is well received by our students and their appraisal is presented on page 3.

It was very pleasing to see the Biozentrum with its strong post-graduate program ranked #7 in a world-wide competition of best post-doc places outside the US (cf. The Scientist, Vol. 20, No. 3, 2006). It is our continuing goal to build a partnership with the scientific community in industry in Basel and the wider BioValley Basel area. Sponsored by Novartis Pharma International, we have organized, on behalf of the University of Basel a series of lectures of Nobel Laureates, which were widely advertised and usually attracted an audience of 300–500 participants. So far, we have had the privilege to receive Prof. Pierre-Gilles de Gennes (Nobel Laureate in Physics, 1991), Thomas R. Cech (Nobel Laureate in Chemistry 1989), Günter Blobel (Nobel Laureate in Medicine 1999), Stanley B. Prusiner (Nobel Laureate in Medicine 1997), Mohamed ElBaradei (Nobel Peace Prize 2005), James D. Watson (Nobel Laureate in Medicine 1962) and Paul Nurse (Nobel Laureate in Medicine 2001).

A major event was the strong support by the Werner-Siemens Foundation for a graduate Program. In 2007 the Biozentrum launched its new PhD program “Opportunities for Excellence”. By means of a generous financial contribution from the “Werner Siemens-Foundation, Zug” up to ten fellowships are awarded each year on a competitive basis. The program aims at selecting and promoting highly qualified students in the area of Life Sciences, encourages selection and excellence at a public university, and strives to strengthen the research landscape in Basel. The fellowships offer direct access to the Biozentrum PhD program, generous financial support, a rotation-based selection of the research group, and support for the attendance of scientific meetings and courses during the PhD. From a total of 350 applications that were received from all over the world in 2007, 23 students were short listed and invited for a two-day interview. From these the PhD committee, which represents all five focal areas and core programs of the Biozentrum, selected the first nine fellows of the program. For further information see: http://www.biozentrum.unibas.ch/phd

The Biozentrum is also intensely engaged in the SystemsX initiative: The ETH Zurich, the University of Zurich and the University of Basel will collaborate in the latest highly interactive scientific approach to biological research: Systems Biology. At least one new department of this cross-disciplinary faculty will be located in Basel. In addition, the Biozentrum will participate in the SystemsX program by contributing several scientific programs.

J. Seelig

Joachim Seelig, Chairman Biozentrum
January 2008

Teaching Activities

The Biozentrum educates scientists at the bachelor, master, doctoral and postdoctoral level. The particular advantage of the education at the Biozentrum is its direct integration into research. All lecturers are active, grant holding scientists. The ratio of lecturers to students is ideal (although the student numbers are gradually increasing) and ensures optimal support for every student.

Bachelor in Biology

In 2003 the current biology curriculum was adjusted to the Bachelor/Master system according to the Bologna convention. The Bachelor curriculum takes three years to complete and is taught by lecturers of the Biozentrum and the Department of Integrative Biology. During the first two years of studies, the students receive the essential basics in mathematics/statistics, physics, and chemistry, as well as introductory courses in biology. In addition, they are trained in ethics and attend optional courses in biology and non-biological subjects. After having completed the basic studies, the students must choose either molecular biology, organismic biology or integrative biology to be the main focus of their further studies. The content of the third year, which consists of practical courses lasting several weeks each, determines the Major of the Biology Bachelor. The block courses provide theoretical information which the students then turn into practice in the laboratory or in the field.

Master in Molecular Biology

Students with a Bachelor in Biology, Major Molecular Biology, are admitted to the fast track Master program that takes 1 to 1½ years. The students work primarily on their Master thesis and take additional courses in molecular biology in their particular filed of specialization. The Master thesis is the students' real introduction to research. At the Biozentrum, the students can specialize in Biochemistry, Bioinformatics, Biophysics, Cell Biology, Developmental Biology, Genetics, Immunology, Infection Biology, Microbiology, Neurobiology, Pharmacology, and Structural Biology. A list of the Master & Diploma theses completed during the report period can be found on pp 5–6. The interaction between students and the lecturers is additionally supported by the so-called career mentoring: at the beginning of their studies, every student is assigned to a certain lecturer, which implies an early contact between researchers and students. This lecturer, the career tutor, is the student's contact person and personal advisor during the entire duration of his/her studies.

The PhD program

The Biozentrum offers an international Graduate Teaching Program in different areas of Molecular Life Sciences. Students accepted to the program conduct their own research project and attend scientific and soft skill lectures offered by the Biozentrum Graduate Teaching Program. The new teaching program features lecture cycles covering infection biology, neuroscience, cell biology, development, structural biology and biophysics, computational and systems biology, plant sciences, molecular biology, and molecular medicine. Students are expected to finish their PhD in 3–4 years. After approval of the dissertation by the Natural Science Faculty of the University of Basel, the studies are concluded with a detailed oral examination. A list of the dissertations completed during the report period can be found on pp 7–10.

In order to promote excellent young scientists, in 2007 the Biozentrum, together with the Werner Siemens-Foundation, has launched the graduate study program „Opportunities for Excellence". The program offers 10 PhD fellowships per year on a competitive basis with direct access to the PhD program, generous financial support and a rotation-based selection of the research group and support for the attendance of scientific meetings and courses during the dissertation.

Seminars at the Biozentrum

The Biozentrum has several seminar series, organized by the focal areas and the core programs, planned several months in advance and generally given by a senior scientist. Many additional seminars are given that cannot be fitted into the formal series either because they are arranged on short notice or because of scheduling conflicts. We call these "Informelle Seminare", informal seminars. They are, however, just as widely announced as the formal ones and many speakers are surprised to find themselves giving their informal seminar to a packed auditorium. These seminars play an important role in the life of the Biozentrum. They are perhaps the most important post-graduate educational activity that we offer, and many a graduate student has secured his first postdoctoral position while sharing a post-seminar drink with the speaker.

Students' Council Report
Verein Studierender der Biologie
VSB

Studying Biology at the Biozentrum in Basel – an enriching and challenging experience

The opportunity to accomplish our Bachelor's degree in either "Molecular Biology", "Integrative Biology" or "Animal and Plant Sciences" enables us students of biology at the University of Basel to take a first step towards our area of preference during undergraduate education. The curricular concept to separate organismic and molecular biology, which is mirrored in the independence of the Biozentrum as an individual department, appeals to most students, as they hold interests as rich and various as the enormous diversity of subtopics in biology. For those seeking to combine organismic and molecular fields, the faculty offers the academic freedom to study "Integrative Biology" – a mixture of the two mentioned orientations. For many students, the free and still rather broad options to design the curriculum according to personal preferences positively contribute to the dwindling philosophy of an education in the pursuit of knowledge for its own ends rather than for economic purposes.

During the first two years, we are trained in basic scientific disciplines adjacent to biology such as math, physics and chemistry, including some practical work. This provides the opportunity to acquire the basic concepts and tools of scientific thinking. In addition, students gather a broad theoretical background knowledge in biology, as we attend – regardless of later specialisation – the same basic lectures in the initial four semesters. These cover fields reaching from animal and plant sciences or ecology to neurobiology, structural biology or biochemistry.

The third year is organised in practical block courses, a concept that seems popular among a large majority of students. It has been repeatedly pointed out that the block courses provide an excellent opportunity to engage in a particular subject much more thoroughly than any lecture would allow: The combination of practical and theoretical work seems to play a key role in understanding biology far beyond the limited profoundness which theoretical teaching can mediate alone. But apart from helping to understand complex biological mechanisms and pathways, the numerous experiments in the block courses also enable students to develop valuable practical skills.

Furthermore, a factor always important to students is the assistant-to-student ratio, which reaches the value of 1:3 during some block courses, allowing almost everyone to have his or her own personal trainer. Although block courses mean very hard work at the time for many students, they are retrospectively perceived as well-designed and intelligent establishments, connecting theoretical and practical aspects and giving students a highly valuable first insight into the actual world of science and research.

The basic background taught in the four initial semesters and the block courses complement each other in preparing us for the Master's degree. During graduate studies, students have to prove their abilities to work as scientists in one of the various Biozentrum labs. Supervision by advanced students provides guidance through the whole year to ensure good results in the Master thesis. Generally, graduate students seem to draw a positive personal conclusion from their academic education at the Biozentrum.

The fact that each step of our curriculum logically builds on the previous one, shows how important it is to ensure a high quality of teaching not only on the advanced level of graduate studies, but also on an entirely basic level from the very beginning of every academic education. Therefore, we must strongly emphasise the importance of didactics in teaching and would like to encourage the staff at the Biozentrum to give careful consideration to this aspect so important to students. This could be, for instance, increasingly done by establishing a quality-control programme for didactics in collaboration with the students' council. Awareness of a high quality standard in teaching – traditionally probably more pronounced in the Anglo-American academic world – constitutes an important part of academic excellence of many of the word's leading universities and becomes increasingly weighted in several international academic ratings. Indeed, we agree that a didactically well-organised lecture may open doors which books alone sometimes fail to do.

All in all, we conclude that studying biology at the Biozentrum in Basel stands for a solid, interesting and challenging academic education of high quality. In this context, the uniqueness of the block courses should be em-

phasised once more: In addition to their valuable academic function, they mediate a strong practical background leading to advantages for graduate studies and, as a consequence, potentially in the job market.

Apart from the mentioned benefits which the Biozentrum at the University of Basel holds in contrast to other Swiss universities, however, prospective students should not forget either that Basel is an interesting place to live: As a culturally rich international hotspot, just a few minutes away from the French and German border, it is not surprising that one meets people from different countries in Basel and international students of various background at the Biozentrum. The exchange with a foreign student is often an experience of both personal and scientific enrichment.

For questions or comments: Simon.Staehli@stud.unibas.ch

Diploma Theses & Masters

*Althaus, Jasmin: *Notch signaling in mouse and human T cell development and Recombinant BAFF rescues mature B cell development in baff deficient mice*. (A. Rolink, C. Arrieumerlou)

Aregger, Regula: *Cloning expression, purification and NMR-characterization of two small model peptides*. (S. Grzesiek)

Beck, Andreas: *Quantitative modelling of bio-molecular interactions by isothermal titration calorimetry*. (H. Heerklotz/J. Seelig)

*Bennion, Janis Michelle: Drosophila Genetics as a tool in search for novel components of the kinase signaling pathway. (M. Affolter, G. Thomas)

Betz, Charles: *Transformation of mast cells to IL-3 independent growth by downregulation of TSC2 and activation of the TOR pathway*. (Ch. Moroni & M. Hall)

*Bläuer, Ueli: *Establishment and Phenotypic Analysis of a N-Degron Based Conditional NEJ2 Mutant in Saccharomyces cerevisiae.* (P. Schär, M. Affolter)

Blum, Yannick: *Development of the intersegmental Vessel in the zebrafish: an analysis at the cellular level*. (M. Affolter)

*Boller, Silvia Cornelia: *Large-scale flux analysis of transcriptional regulator mutants on Saccharamyces cerevisiae.*(S.-M. Fendt, U. Saurer, M. Affolter)

*Chang, Lena: *Identification of possible biomarkers to study the aging process in C.elegans.* (Z. Alcedo, M. Affolter)

*Cremonesi, Alessio: *Prediction and Profiling of clinical resistance parameters of a novel HIV protese inhibitor*. (T. Klimkait, M. Affolter)

*Egli, Samuel: *Polyethoxylated surfactants: Interaction with the multidrug transporter P-glycoprotein*. (FHBB Muttenz/A. Seelig)

* Ewald, Collin: *Growth Control and Epigenetics Research Seminar.* (J. Alcedo, M. Affolter)

*Fiechter, Chantal: *Study of the resistance of Capnocytophaga canimorsus to the killing action of complement*. (G. Cornelis, G. Pluschke)

Finlayson, Mark: *Comparing the gene order in Ashbya gossypii and Saccharomyces cerevisiae to study genomic rearrangement events. The effects of disrupting AgCDC15, AgDBF2/20 and AgMOB1 in Ashbya gossypii*. (P. Philippsen)

*Flückiger, Patrick: *The role of CAP23 in development and function of the immune system.* (A. Rolink, M. Affolter)
Gerber, Martha: *Designing Diguanylate Cyclase Inhibitors*. (M. Christen & U. Jenal)

*Heimgartner, Fabienne: *Immune homeostasis in cytokine transgenic mice.* (D. Finke, M. Affolter)

Herwig, Lukas: *Analysis of ISV architekture in the zebrafish embryo of the cellular level by different tools*. (M. Affolter)

*Hilbert, Manuel: *Charakterisierung und Fluoreszenzmarkierung der aus zwei Domänen rekonstituierten reversen Gyrase aus Thermotoga maritima*. (D. Klostermeier)

Huber, Roland: *Molecular Mechanisms controlling Aspects of Motor System Development in Mouse*. (S. Arber)

Huser, Sonja: *Tools for the detection of the B.heselae VirB/VirD4 secretion system*. (Ch. Dehio)

Jaggi, Sandra: *The cholesterol regulation pathway as a readout for COPII-dependent vesicle transport*. (H.P. Hauri)

*Karow, Anne: *Rekonstitution der RNA-Helikase YxiN durch Intein-vermittelte Verknüpfung der Helikasedomänen und Entwicklung von Strategien zur ortsspezifischen Markierung mit zwei Fluoreszenzfarbstoffen*. (D. Klostermeier)

Käslin, Fabian: *The role of Mdm4 in cancer*. (J. Hergovich Lisztwan, D.Fabbro, H.P. Hauri)

*Kohler, Götz: *Die Laktat-Aufnahme-Kapazität menschlicher Erythrozyten und die körperliche Leistungsfähigkeit*. (MAS Medizinphysik, ETH Zürich/ J. Seelig)

*Kohler, Reto: Search for physiological substrates of the NDR protein Kinase. (B. A. Hemmings, M. Affolter)

Küng, Raphael: *Dissection of the Zinkfingerprotein Schnurri and ist molecular function.* (M. Affolter)

*Liu, Kun: Genetic Studies of Familial Myeloproliferative Disorders. (M. Affolter, R. Skoda, A Pappassotiropoulos)

*Lüdi, Ursula: *Promiscuous gene expression in thymic epithelial cells during graft-versus-host disease*. (G. A. Holländer, U. A. Meyer)

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*Mathys-Schneeberger, Arina: *The role of microRNAs in posttranscriptional regulation of ULBP1, an activating ligand for human natural killer cells*. (Aleksandra Wodnar-Filipowicz, J. Pieters)

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Müller, Christian: *Thermodynamics of the random coil to β-sheet transition of amyloid β-peptide in a membrane environment*. (J. Seelig)

Nyffenegger, Christian: *Dynamics in different states of Parvalbumin, measured by Triplet-Triplet energy transfer*. (T. Kiefhaber)

Odathekal, Juliet: *Highlighting the septation process in the filamentous fungus Ashbya gossypii*. (P. Philippsen)

*Osterwalder, Marco: *Function of Hand2 in early limb bud polarisation*. (A. Galli, R. Zeller, M. Affolter)

Perisa Damir: *Characterization of the function of the mycobacterial Serine/Threonine Protein Kinase G in vivo and ist phylogenic relationship to other Serine/Threonine Kinases* (J. Pieters)

*Roth Franziska: *Regulation of BAFF receptor expression during B cell activation*. (A. Rolink, S. Arber)

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*Schulz zur Wiesch, Pia: *Mapping of fibronectin binding sequences in tenascin-C* (G. Christofori, M. Ruegg)

Simon, Susanne: *Components of cyclic-di-GMP signaling and their role in the small colony variant morphotype of Pseudomonas aeruginosa*. (J. Malone, U. Jenal)

Steiner, Samuel: *Molecular mechanisms of Escherichia coli biofilm induction by translational inhibitors*. (A. Böhm & U. Jenal)

Stutz, Cian: *The crystal structure of ecYbiB - Insight into the evolution of novel protein functions*. (O. Mayans)
*Truttmann, Matthias: *Inhibition kinetics of TEM-type class A β-lactamases.* (M.G. Page, U. Jenal)

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*Ziegler, Dominik: Transcription factors function during epithelial-to-mesenchymal transition (EMT). (G. Christofori, M. Affolter)

*Zoller, Frank: *Nano-Structured Substrates for Single Cell Proteomics*. (A. Engel)

* work performed outside of the Biozentrum

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Anastasiou, Dimitrios: *Identification of novel mechanisms regulating the NAD+-dependent deacetylase SIRT1*. (M. Hall, W. Krek, M. Peter)

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*Kaeser, Patrick: Analysis of in vivo functions of Memo in embryonic and mammary gland development. (N. Hynes, P. Matthias, M. Affolter)

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*Murigande, Claire: *Synergistic and antagonistic effects of TNFα and IGF-I in heart failure: in vitro and in vivo study of cardiac and skeletal muscle*. (A. Eberle, M. Brink, K. G. Hofbauer)

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*Petrimpol, Marco: *Hypoxia-induced signaling in Angiogenesis. Role of mTOR, HIF and Angiotensin II*. (K. Hofbauer, E. Battegay, M. Brink)

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Rhomberg, Thomas Alexander: *Molecular and Cellular Baseis of the Internalization of Bartonella henselae by Human Endothelial Cells*. (Ch. Dehio)

Ruiz, Christian: *Identification and validation of novel amplification target genes in human breast cancer.* (M.A. Rüegg, G. Sauter, R. Chiquet-Ehrismann)

*Santos Ferrao, Alexandre: *Regulation of anatomical plasticity at neuromuscular junctions. (*Pico Caroni / Silvia Arber)

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Focal Area
Growth & Development

Research at the Biozentrum in the area of "Growth and Development" is dedicated to understanding the molecular basis of life. How do biomolecules interact to ultimately constitute a living organism? To understand these interactions at a quantitative and atomic level, members of the focal area collaborate extensively with others at the Biozentrum, in particular with members of the core programs "Structural Biology and Biophysics" and "Computational and Systems Biology". As described below, the research programs of the individual groups in the area "Growth and Development" focus on membrane and protein traffic, signal transduction, gene regulatory networks and RNA processing, in a wide variety of experimental organisms ranging from yeast to mammals.

Our focal area comprises nine research groups, headed by Profs. Markus Affolter, Walter Gehring, Michael Hall, Hans-Peter Hauri, Urs Jenal, Walter Keller, Peter Philippsen, Anne Spang, and Martin Spiess. Prof. Anne Spang joined the Biozentrum in 2006 and is already fully integrated in the research and teaching efforts at the Biozentrum. With her highly energetic participation in all aspects of the focal area, she is indeed a highly appreciated addition. Another focal area highlight within the last two years has been the significant enhancement of our live imaging capabilities, due to the fund-raising efforts of Prof. Markus Affolter. Prof. Walter Gehring was awarded an honorary doctorate (Dr. h.c) from the Pierre & Marie Currie University in Paris for his seminal discoveries in developmental biology. Prof. Michael Hall was elected to the Swiss National Science Foundation Research Council. Prof. Hans-Peter Hauri was elected Dean of the Faculty of Natural Sciences. Prof. Urs Jenal established and heads the Werner Siemens Foundation PhD Program at the Biozentrum. Furthermore, the students and postdoctoral fellows within the focal area continue to be successful in their career advancement with, for example, Stepanka Vanacova from the Keller laboratory receiving an EMBO Installation Grant to establish her own laboratory in Brno, Cech Republic. Matthias Christen from the Jenal laboratory was awarded the Swiss Society for Microbiology Award 2007.

All members of the focal area are heavily involved in teaching at the undergraduate and postgraduate levels, including introductory and advanced lecture series. The graduate teaching program at the Biozentrum consists of nine topic-oriented cycles with a total of almost 50 courses. Peter Philippsen also teaches extensively in the Biotechnology curriculum, organized by the "Upper Rhein Universities" in Basel, Strasbourg, Karlsruhe and Freiburg/Breisgau.

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Focal Area
Growth & Development

Seminars by invited speakers are organized on a regular basis, in particular through the well-attended "Growth and Development" seminar series that hosts internationally prominent scientists.

Raphael Küng, Roman Lutz*, Flora Mauch*, Cédric Meury, Dominique Meyer, Juliet Odathekal*, Susanne Schär*, Marie-Joelle Schmidt, Susanne Simon*, Dominik Sutter, Andreas Stalder, Samuel Steiner, Patrick Tschopp*, Irene Wiederkehr*, Jeannine Winkler*, Wiebke Wlotzka, Barbara Zollinger

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Cell signalling and cell rearrangement during organ morphogenesis

The organization of body pattern in developing multicellular organism is controlled to a large extend by cell-cell signaling. Great efforts have been devoted in the past decades to identify and characterize the molecular components of a relatively small number of diverse developmental signaling cascades conserved throughout evolution. We have been studying two important developmental signals (Dpp/BMP and Fgf), and our efforts concentrated first on characterizing the signaling pathways in detail and decipher their molecular logic, and second on understanding how these pathways control exquisite cellular behavior during development, both in Drosophila and in zebrafish. Our most intense research efforts are directed towards a profound understanding of cell behavior in branching morphogenesis, a process that leads to the ramification of epithelial structures such as seen in the lung, the kidney, many internal glands as well as the vascular system.

Cell signaling in organ formation

It has been proposed more than a century ago that the organization of body pattern might be controlled by so-called morphogen gradients. Only recently has it been possible to demonstrate that secreted proteins of the Transforming Growth Factor β (TGFβ), Wnt and Hedgehog families specify positional information by this mechanism. Drosophila Dpp is a member of the TGFβ superfamily and was the first secreted protein for which a morphogen function has been clearly demonstrated. Over the past ten years we have characterized the Dpp signaling pathway in detail, in collaboration with the group of Konrad Basler in Zurich. Surprisingly, two nuclear proteins, Schnurri (Shn) and Brinker (Brk), play major key roles in Dpp signaling and are responsible for much of Dpp's function as a morphogen. Brk is a DNA-binding transcription factor and acts as a default repressor; Brk represses Dpp target genes prior to ligand-induced receptor activation. Loss of Brk function causes overproliferation of imaginal discs and ectopic expression of Dpp target genes, while gain of Brk function causes a general loss of Dpp signaling. We find that throughout development, Dpp causes the dose-dependent transcriptional downregulation of the *brk* gene, and it turns out that the inverse gradient of Brk generated by the Dpp gradient is instructive and essential for patterning and organ development; e.g. the cells read out Dpp signaling levels primarily at the *brk* gene, and Brk protein then acts as a dose-dependent repressor to instruct the cell with regard to its position within the gradient.

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Markus Affolter

Since our studies showed that the conversion of an extracellular Dpp gradient into an inverse transcriptional gradient of *brk* is the most important step in morphogen readout, we concentrated our efforts in understanding the events involved in *brk* repression at the molecular level. The dissection of the *brk* regulatory region identified two elements with opposing properties involved in the morphogen readout, a constitutive enhancer and a Dpp morphogen-regulated silencer. We found that the *brk* silencer serves as a direct target for a protein complex consisting of the Smad homologs Mad/Medea (which transduce the Dpp signal to the nucleus) and the zinc finger protein Schnurri. Together, these results provide the

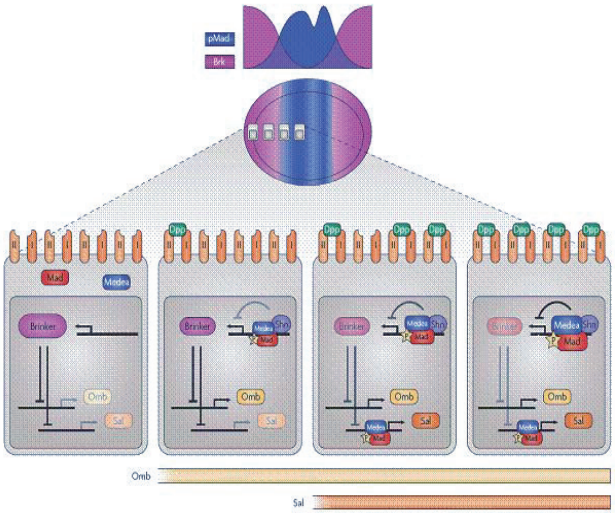

Figure 1: The graded distribution of the Dpp ligand leads to differences in the activation levels of the Punt/Tkv receptor complex, and ultimately to decreasing levels of pMad/Medea with increasing distance to the Dpp source. The amount of pMad/Medea is sensed at the silencer elements in the brk locus, and via the recruitment of Shn, brk transcription is gradually repressed by increasing concentration of pMad/Medea. This mechanism leads to the formation of a gradient of Brk in which the levels of Brk are inversely correlated to the amount of pMad/Medea. Brk levels are important to set the expression boundaries of target genes such as omb and sal. Taken from Affolter and Basler (2007) Nat Rev Genet. 9, 663-74.

molecular framework for a mechanism by which the extracellular Dpp morphogen establishes a finely tuned, graded read-out of a transcriptional repressor complex (Figure 1). Other morphogens, which pattern the nervous system or the limb fields in higher vertebrates, might use similar mechanisms. Our results also demonstrate that developmental signalling pathways can actively repress signalling in a ligand-induced manner. It remains open to what extend other developmental signalling pathways can also repress gene transcription, and whether they use similar mechanisms. Our current efforts are devoted to a systems biology approach, involving genome-wide target gene identification, real time analysis of morphogen gradient readout, and computer modelling to better understand the dynamcs of the Dpp morphogen system. These studies will eventually lead to a comprehensive understanding of morphogen function in tissue growth and patterning, a key issue in modern developmental biology.

Cell rearrangement in organ formation

To gain insight into how signaling pathways control more complex cellular decisions during the process of organ morphogenesis, we investigate the formation of the Drosophila tracheal system, an epithelial branched network similar to the lung, the kidney or the vasculature. Tracheal development serves as a paradigm to understand how epithelial cell sheets can be transformed by cell signaling and cell-cell or cell-matrix interactions into complex three-dimensional networks, a process generally referred to as branching morphogenesis. Our approach has been to identify genes involved in the process by genetic analysis, and the characterization of relevant gene products by *in vivo* and *in vitro* analysis. In addition, we have devoted major efforts to characterize branching morphogenesis at the cellular level, using avant-garde live imaging technology. Over the past decade, these studies have provided a framework for understanding complex processes involved in the architectural design of developing organs, including the control and integration of cell migration and cell rearrangement via cell-cell signaling and extracellular matrix components (Figure 2).

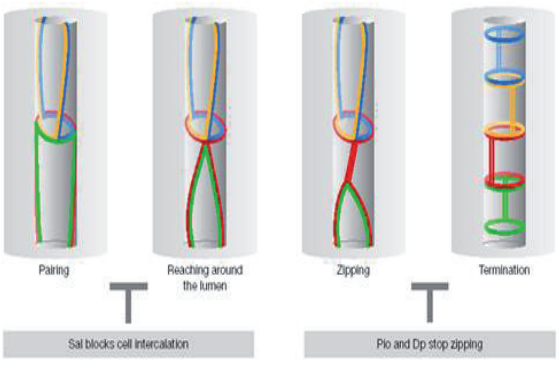

Figure 2: Steps of adherence junction remodelling during cell intercalation and tube elongation. For details, see Neumann and Affolter (2006) EMBO reports 7, 36-40.

Markus Affolter

Studies on the development of blood vessels in higher organisms suggest strong parallels between tracheal development in insects and tube formation in the growing vasculature. Interested by this possible developmental similarity, we have initiated studies aimed at a better understanding of blood vessel development in zebrafish, one of the most promising animal systems in the study of angiogenesis available at the moment. We indeed find that our approach to study cell rearrangement during tracheal development provides novel insight into how cells behave during angiogenesis when applied to zebrafish. We have recently proposed a novel model for the architecture of the first vessels formed via angiogenesis, a model which is strikingly different to the one previously described (see Blum *et al*., 2007). Our studies re-define the cellular routines involved in angiogenesis, and provide the basis for all future studies in the zebrafish regarding angiogenesis. We have now strengthened our efforts to study angiogenesis using live imaging combined with novel transgenic lines and strategies.

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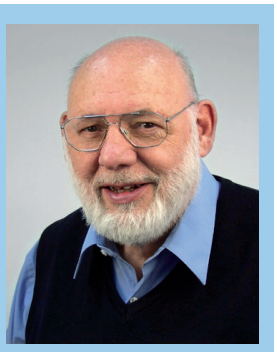
Hox and Pax, Master Control Genes in Development and Evolution

The body plan of animals and humans is specified by master control genes which have been highly conserved during evolution. Despite of a bewildering variety of modes of development, ranging from organisms with a fixed cell lineage to animals in which the cell lineage is highly variable, the Hox genes which control the differentiation along the anterior-posterior axis have been conserved during evolution, not only in their DNA sequences, but even in their ordered arrangement along the chromosome. The "head" genes being located at the 3'end of the Hox cluster and the "tail" genes at the 5'end with the intermediate genes being alined on the chromosome in the same order as they are expressed along the anteroposterior body axis. This phenomenon is designated as colinearity.

The evolution of the Hox gene cluster and the notion of a developmental ground state

We have extended the model proposed by E.B. Lewis for the Bithorax Complex to the entire Hox complex including the Antennapedia Complex with the anterior thoracic and the head genes. The ordered colinear arrangement of the genes can be explained by the mechanism of unequal crossingover which leads to progressive gene duplications starting from an urhoxgene. Our phylogenetic analysis suggests that the most ancient genes are Hox 1 and Hox 9 (or 13) located at either end of the cluster and that the genes in the middle of the cluster have retained the greatest similarity to urhoxgene. We propose that the urhoxgene and its descendents (Hox 6) specify and maintain the developmental ground state. We define the developmental ground state on the basis of the homeotic mutations. Loss-of-function mutations lead back to the ground state, whereas gain-of-function mutations lead away from the ground state. These mutations have best been studied in Drosophila where they are associated with well defined morphological transformations. The developmental ground state is specified in Drosophila by the **Antennapedia** gene (Hox 6) which specifies the second thoracic segment T2 with a pair of second legs and pair of wings. Evolutionary considerations indicate that dipteran insects originated from ancestors which had legs on all thoracic and abdominal segments (like certain crustaceans) and two pairs of wings (like the more primitive insects). The posterior Hox genes have evolved to convert the second pair of wings into halteres, and to remove the legs from the abdominal segments. Loss-of-function mutations in *Ultrabithorax* (Ubx) lead to the transformation of halteres into wings, back to the ground state, whereas gain-of function mutations lead to the transformation



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of wings into halteres (away from the ground state). Similarly, loss-of-function mutations in the **Abdominal-B** (Abd-B) gene lead to the formation of legs on the most posterior abdominal segments, whereas gain-of-function mutations convert all thoracic and abdominal segments to the last abdominal segment A8. In the anterior part of the fly the mouthparts like the maxillary palps and the labial palps are thought to represent modified legs, gnathal legs. Loss-of-function mutations in the proboscipedia gene lead to the transformation of the distal proboscis to thoracic legs (towards the ground state), whereas gain-of-function mutations convert the adult legs into maxillary or labial palps (away from the ground state). This defines the ground state as being in the middle of animal, in the second thoracic segment.

Epistatic relationships between the various Hox genes

We have previously shown that Antennapedia (Antp) is repressed by the next posterior Hox gene *Ultrabithorax (Ubx)* and that this posterior repression (epistasis) occurs at the transcriptional level. However, it soon became obvious that transcriptional crossregulation cannot explain all of the epistatic interactions. Yoshitsugu Adachi has analyzed the epistasis of *Abdominal-B* (*Abd-B*) over *Antp* and shown by Bimolecular Fluorescence Complementation (BFMC) that ABD-B and ANTP proteins interact *in vivo* in imaginal discs. By attaching the N-terminal half of Venus (a strongly fluorescent GFP derivative) to the AbdB homeodomain and the C-terminal half to the Antp homeodomain he obtained strong nuclear fluorescence when the two homeodomains interact. Therefore, the epistasis of *Abd-B* over *Antp* is due homeodomain-homeodomain interactions.

DNA binding properties of the Antennapedia homeodomain as studied by Fluorescence Cross-Correlation Spectroscopy (FCCS) on single molecules

The binding of the Antp homeodomain to its DNA target sites has been studied by FCCS. The homeodomain was labeled by adding a tetracysteine motif to which a fluorescent dye, lumiogreen, can be covalently attached. This so-called FIAsH technology was used successfully to measure the binding constants of the homeodomain to Cy5-labeled DNA in single molecules in solution by FCCS and to compare the results with those obtained by gel mobility shift assays (EMSA). The dissociation constant K_D was found to be in the same nanomolar range as determined previously by EMSA. However, the half-life of the DNA-protein complex in solution is by several orders of magnitude shorter than in the gel (Beaufils *et al*., submitted).

Pax genes and the evolution of eyes and photoreceptors

Our earlier studies have identified the Pax6 gene as a master control gene for eye morphogenesis. Pax6 orthologs have been found in all bilaterean animals analyzed, and for many we have shown that they can substitute for the Pax6 homolog *eyeless* (ey) in Drosophila. However, in the more primitive cnidarians and sponges no bona fide Pax6 gene has been found. Jellyfish and corals have four Pax genes: A, B, C and D only. In the jellyfish Tripedalia which has highly developed eyes the PaxB gene was found to be capable of inducing ectopic eyes in Drosophila by Kozmic *et al*. The PaxB gene of Tripedalia might be a precursor of Pax6 since it has a homeobox similar to Pax6 and a pairedbox more closely related to Pax2, the next “relative” of Pax6. However, in the hydrozoan jellyfish Cladonema we found that PaxA rather than PaxB is capable of inducing ectopic eyes in Drosophila. This difference between the two classes of jellyfish remains to be analyzed further. In tracing back the Pax genes to the sponges my postdoctoral fellow Hiroshi Suga has found a single gene in the Ephydatia, a fresh water sponge, as well as in a marine sponge *Microciona*. Interestingly, this single pax gene is more similar to Pax2 than Pax6, but it is capable of inducing eyes in Drosophila.

Multiple Opsin genes in Cladonema

Hiroshi Suga carried out the first analysis of opsin genes in jellyfish, in Cladonema, a species with well developed eyes and in *Podocoryne* a species lacking eyes. Much to our surprise it turns out that *Cladonema* has as many as 18 opsin genes of which only seven are expressed in the eyes. *Podocoryne* which has no eyes, nevertheless has two opsin genes. Besides the opsin genes expressed in the eyes, *Cladonema* has opsin genes which are specifically expressed in the tentacles, in the manubrium and some that are expressed ubiquitously. Among those expressed in the manubrium, some are expressed in the gonads specifically in the oocytes. This is of particular interest. Since PaxB is also expressed in the gonad, which could indicate that the gonad is capable of sensing light. This might provide an explanation for the fact that jellyfish, even those without eyes, spawn seasonally. The functional significance of these findings remains to be analyzed further (Suga *et al*., submitted).

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TOR signaling and control of cell growth

Introduction

Cell growth is highly regulated. Cells respond to nutrients or other appropriate growth stimuli by up-regulating macromolecular synthesis and thereby increasing in size. Conversely, cells respond to nutrient limitation or other types of stress by down-regulating macromolecular synthesis and enhancing turnover of excess mass. Thus, the control of cell growth involves balancing positive regulation of anabolic processes with negative regulation of catabolic processes. Growth is also controlled relative to cell division. In proliferating cells, growth is linked to the cell cycle such that most cells precisely double their mass before dividing. In other physiological contexts, such as load-induced muscle hypertrophy or growth factor-induced neuronal growth, cell growth is controlled independently of the cell cycle. Furthermore, in addition to the temporal control of cell growth described above, cell growth can be subject to spatial constraints. For example, budding yeast and neurons grow in a polarized manner as a result of new mass being laid down only at one end of the cell. Finally, in multicellular organisms, growth of individual cells is controlled relative to overall body growth such that the organs and tissues constituting the organism are properly proportioned.

The TOR signaling network

What are the mechanisms that mediate and integrate the many parameters of cell growth? In other words, what determines that a cell grows only at the right time and at the right place? Remarkably, the study of these mechanisms has been largely neglected, despite their clinical relevance and despite cell growth being, along with cell division and cell death, one of the most fundamental (and obvious) aspects of cell behavior. Also remarkable is the finding that cell growth control, regardless of eukaryotic organism or physiological context, seems always to involve the protein kinase TOR (Target Of Rapamycin) and its signaling network. TOR has thus become known as a central controller of cell growth. Indeed, the discovery of TOR led to a fundamental change in how one thinks of cell growth. It is not a spontaneous process that just happens when building blocks (nutrients) are available, but rather a highly regulated, plastic process controlled by TOR-dependent signaling pathways. TOR, originally discovered in our laboratory, is structurally and functionally conserved from yeast to mammals (including worms, flies, and plants). TOR in mammals (mTOR) controls cell growth and metabolism in response to nutrients (e.g., amino acids), growth factors (e.g., insulin, IGF-

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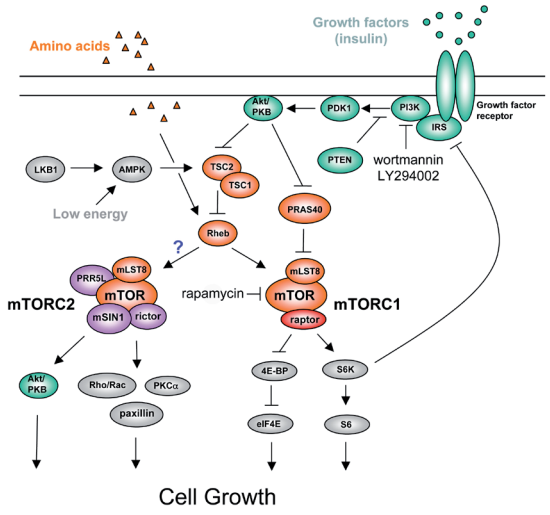
1, PDGF), and cellular energy status (ATP). Nutrients are the dominant TOR input as high levels of amino acids can compensate for an absence of the other mTOR inputs but not vice versa, and only nutrients activate TOR in unicellular organisms. The growth factor signaling pathway, grafted onto the more ancestral nutrient sensitive TOR pathway, co-evolved with multicellularity. TOR activates cell growth by positively and negatively regulating several anabolic and catabolic process, respectively, that collectively determine mass accumulation and thus cell size. The anabolic processes include transcription, protein synthesis, ribosome biogenesis, nutrient transport, and mitochondrial metabolism. Conversely, TOR negatively regulates catabolic processes such as mRNA degradation, ubiquitin-dependent proteolysis, autophagy and apoptosis. TOR is an atypical serine/threonine kinase that is found in two functionally and structurally distinct multiprotein complexes, TORC1 and TORC2 (mTORC1 and mTORC2 in mammals), each of which signals via a different set of effector pathways. TORC1 is rapamycin sensitive whereas TORC2 is rapamycin insensitive. The best-characterized phosphorylation substrates of mTOR are S6K and 4E-BP1 via which mTORC1 controls translation, and Akt/PKB via which mTORC2 controls cell survival and likely other processes. Like TOR itself, the two TOR complexes and the overall architecture of the TOR signaling network are conserved from yeast to human. As a central controller of cell growth and metabolism, TOR plays a key role in development and aging, and is implicated in disorders such as cancer, cardiovascular disease, obesity, and diabetes.

We are studying the TOR signaling network in the yeast *Saccharomyces cerevisiae* and in mammalian cells. A major finding in our laboratory in recent years was the fact that TOR controls cell growth via two major signaling branches. Furthermore, we discovered the two TOR complexes and demonstrated that these two complexes correspond to the two previously described signaling branches. Examples of more recent studies on the TOR signaling network and cell growth control in yeast and mammalian cells are as follows.

Growth and stress are generally incompatible states. Stressed cells adapt to an insult by restraining growth and, conversely, growing cells keep stress responses at bay. This is evident in many physiological settings, including for example the effect of stress on the immune or nervous system, but the underlying signaling mechanisms mediating such mutual antagonism are poorly understood. Calcineurin



TOR signaling network in mammalian cells

is a conserved, Ca^{2+}/calmodulin-dependent protein phosphatase and target of the immunosuppressant FK506 (tacrolimus) that, in yeast, is activated during stress to promote cell survival. We have shown that yeast mutants defective for TOR complex 2 (TORC2) or the essential, homologous TORC2 effectors SLM1 and SLM2 exhibit constitutive activation of calcineurin-dependent transcription and actin depolarization. Conversely, cells defective in calcineurin exhibit SLM1 hyperphosphorylation and enhanced interaction between TORC2 and SLM1. Furthermore, a mutant SLM1 protein ($SLM1^{\Delta C14}$) lacking a sequence related to the consensus calcineurin docking site (PxIxIT) is insensitive to calcineurin, and *$SLM1^{\Delta C14}$ slm2* mutant cells are hypersensitive to oxidative stress. Thus, TORC2-SLM signaling negatively regulates calcineurin, and calcineurin negatively regulates TORC2-SLM. These findings provide a molecular basis for the mutual antagonism of growth and stress.

In collaboration with Robbie Loewith (University of Geneva), we have described the AGC kinase Sch9 as the first *bona fide* substrate of yeast TORC1. Six amino acids in the C-terminus of Sch9 are directly phosphorylated by TORC1. Phosphorylation of these residues is lost upon rapamycin-treatment as well as carbon- or nitrogen-starvation and transiently reduced following application of osmotic, oxidative or thermal stress. TORC1-dependent phosphorylation is required for Sch9 activity, and replacement of residues phosphorylated by TORC1 with Asp/Glu renders Sch9 activity TORC1-independent. Sch9 is required for TORC1 to properly regulate ribosome biogenesis, translation initiation and entry into G0 phase, but not expression of Gln3-dependent genes.

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Our results suggest that Sch9 functions analogously to the mammalian TORC1 substrate S6K1 rather than the mTORC2 substrate PKB/Akt.

We developed a two-dimensional LC-MS/MS based proteomic strategy to identify new mammalian TOR (mTOR) binding proteins. Using this strategy, we identified the Proline-rich Akt substrate (PRAS40) and the hypothetical protein Q6MZQ0/FLJ14213/CAE45978 as new mTOR binding proteins. Previously identified mTOR binding proteins include Raptor, Rictor and SIN1. PRAS40 binds mTORC1 via Raptor, and is an mTOR phosphorylation substrate. PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 kinase activity toward 4E-BP and PRAS40 itself. HeLa cells in which PRAS40 was knocked down were protected against induction of apoptosis by TNFα and cycloheximide. Rapamycin failed to mimic the pro-apoptotic effect of PRAS40, suggesting that PRAS40 mediates apoptosis independently of its inhibitory effect on mTORC1. Q6MZQ0 is structurally similar to the proline rich protein PRR5 and was therefore named PRR5-Like (PRR5L). PRR5L binds specifically to mTORC2, via Rictor and/or SIN1. Unlike other mTORC2 members, PRR5L is not required for mTORC2 integrity or kinase activity, but dissociates from mTORC2 upon knock down of TSC, a tumor suppressor and negative regulator of mTOR. Hyperactivation of mTOR by TSC knock down enhanced apoptosis whereas PRR5L knock down reduced apoptosis. PRR5L knock down reduced apoptosis also in mTORC2 deficient cells. The above suggests that mTORC2-dissociated PRR5L may promote apoptosis when mTOR is hyperactive. Thus, PRAS40 and PRR5L are novel mTOR-associated proteins that control the balance between cell growth and cell death.

The above describes only part of our recent findings and current research effort. Many other questions concerning the role of TOR and the regulation of cell growth are actively being pursued. For example, we have recently begun studying the role of TOR in the regulation of organismal metabolism. These ongoing studies have interesting implications when considering hormone- and nutrient-related processes such as appetite regulation and aging. Appetite and lifespan are regulated by hormones produced in response to nutrients. However, the nutrient sensor that signals the production of such hormones is not known and may involve TOR signaling.

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Traffic in the secretory pathway

Understanding the molecular mechanisms underlying the secretory pathway is a major goal of cell biology and biomedical research. In eukaryotic cells one third of all newly synthesized proteins are co-translationally inserted into the endoplasmic reticulum (ER) where they are folded, modified and subjected to quality control prior to secretion or transport to various intracellular organelles. The current challenge is to identify all key molecules that catalyze each of the numerous steps in protein sorting and to integrate their function to understand how organelles are formed and maintained with characteristic structure and function and how itinerant proteins are separated from resident proteins. We are studying major questions related to these issues in human and other mammalian cells using live cell imaging, protein fragment complementation and high-throughput siRNA-based gene silencing as well as numerous other cell biological, biochemical, biophysical, and molecular approaches. Knowledge of the fundamental processes controlling the function of the secretory pathway may ultimately lead to new strategies for the treatment of inherited and acquired diseases in which protein secretion is impaired.

Control of the architecture of the early secretory pathway

The secretory pathway of higher eukaryotic cells is composed of the three membrane organelles ER, ERGIC (ER-Golgi intermediate compartment) and Golgi. Maintenance of these organelles requires a balance of anterograde (secretory) and retrograde (backward) vesicular traffic, but how this balance is controlled is poorly understood. Our siRNA-based silencing studies of membrane proteins that cycle between ER, ERGIC and Golgi have revealed that the cargo receptors Surf4, ERGIC-53 and p25 are required to maintain the architecture of ERGIC and Golgi. The molecular mechanism of how these receptors control organelle morphology is currently studied in detail. Another determinant that controls organelle morphology is cargo load. Depending on whether more or less protein needs to be handled, the secretory pathway modulates the size of its organelles. We attempt to identify regulatory factors responding to cargo load.

Dynamics of the ERGIC studied in living cells

The ERGIC defined by the mannose-binding membrane lectin ERGIC-53 consists of a few hundred tubulovesicular membrane clusters and is a mandatory intermediate station for proteins moving from ER to Golgi. Our live imaging studies have conclusively established that the ERGIC clusters constitute stationary membrane entities rather than mobile carriers. Three vesicular pathways depart form the ERGIC (Fig. 1): 1. to the cis-Golgi in

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anterograde direction, 2. to the ER in retrograde direction, and 3. laterally, connecting individual ERGIC clusters. Likewise, the ERGIC receives vesicles from three sides: the ER, the cis-Golgi, and other ERGIC clusters. In studying the mechanisms controlling tri-directional traffic from and to the ERGIC we are focusing on the Arf proteins, a group of small regulatory GTPase, by using a silencing approach.

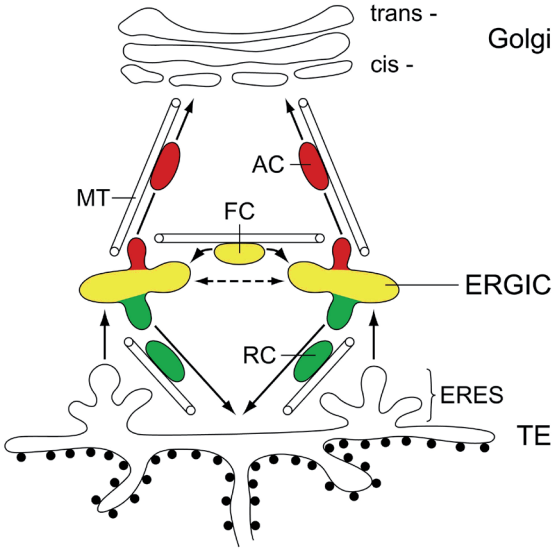

Fig. 1: Model of the organization of the early secretory pathway and sorting of anterograde and retrograde traffic in the ERGIC. AC, anterograde carrier; ERES, ER exit sites; FC, fast-moving carrier; MT, microtubule; RC, retrograde carrier; TE, transitional elelment.

Signal-mediated export of membrane proteins from the ER

Many secretory proteins leave the ER not just passively by default but in a selective way involving transport signals. We found that transmembrane proteins possess cytosolic ER-export motifs interacting with proteins of the COPII coat that shapes transport vesicles forming at ER exit sites. Thus, direct interaction of coat proteins with ER-export signals can actively recruit membrane proteins into transport vesicles. We defined three types of hydrophobic motifs, two of which need to be presented in oligomeric form to operate in ER export, whereas the third one, a C-terminal valine, also operates in monomeric proteins. These signals are recognized by the Sec24 COPII subunit. Human cells express four Sec24 isoforms. Using siRNA-based silencing we studied the role of the Sec24 isoforms in all possible combinations in signal mediated ER-export. The results show that different ER export signals have different preferences for the Sec24 isoforms but there is considerable overlap. We conclude that human Sec24 isoforms expand the repertoire of cargo for signal-mediated ER export, but are in part functionally redundant. Current efforts aim at the identification of the mechanism controlling ER-export of human glycosylphosphatidylinositol-anchored proteins. These proteins become attached to the ER membrane by a lipid anchor, hence do not span the lipid bilayer and cannot directly interact with COPII coat proteins.

Cargo/receptor mechanism of ERGIC-53

The class of leguminous-type lectins (L-type lectins) of the secretory pathway comprises the four type 1 membrane proteins ERGIC-53, ERGL, VIP36 and VIPL, of which ERGIC-53 is most completely studied. ERGIC-53 is a hexameric protein with mannose lectin activity that operates as a cargo receptor mediating efficient transport of some glycoproteins, including cathepsin Z, cathepsin C, and the blood coagulation factors V and XIII. Due to these features ERGIC-53 is an excellent model protein to study cargo receptor-mediated glycoprotein transport early in the secretory pathway. Optimal binding of newly-synthesized glycoprotein cargo to ERGIC-53 in the ER requires high calcium levels and a neutral pH. The precise carbohydrate specificity of ERGIC-53 has long been an enigma. In collaboration with K. Kato's group the carbohydrate specificity of ERGIC-53 has now been established. ERGIC-53 binds high-mannose-type oligosaccharides with low affinity and broad specificity not discriminating between monoglucosylated and deglucosylated high-mannose glycans, very much in contrast to the cycling lectin VIP36 and the ER resident lectin VIPL which have high preference for the deglucosylated A mannose branch. In combination with binding preferences at different pH values, the sugar specificities led to a new model of how the three lectins ERGIC-53, VIPL and VIP36 may operate early in the secretory pathway.

ERGIC-53 forms a complex with MCFD2 (multi coagulation factor deficiency protein 2). We tested the subunit interdependence and cargo selectivity of ERGIC-53 and MCFD2 by siRNA-based knockdown. In the absence of ERGIC-53, MCFD2 was secreted, whereas knocking down MCFD2 had no effect on the localization of ERGIC-53. Cargo binding properties of the ERGIC-53/MCFD2 complex were analyzed *in vivo* using a yellow fluorescent protein fragment complementation assay (YFP-PCA, see below and Fig.2). We found that MCFD2 is dispensable for the binding of cathepsin Z and cathepsin C to ERGIC-53. The results indicate that ERGIC-53 can bind cargo glycoproteins in an MCFD2-independent fashion and suggest that MCFD2 is a recruitment factor for blood coagulation factors V and VIII.

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Combined factor V and factor VIII deficiency

Combined coagulation factor V and factor VIII deficiency is an inherited bleeding disorder in humans due to a defect in either ERGIC-53 or its partner MCFD2. In collaboration with M. Neerman-Arbez and K. Kato we have studied an unusual new case of this disease. Our data show that the patient is a compound heterozygote for two novel mutations in MCFD2, one of which results in the deletion of only 3 amino acids from the C-terminus (deltaSLQ). Biochemical and structural analysis of the deltaSLQ mutant demonstrated impaired binding to ERGIC-53 due to destabilization of the three-dimensional structure of MCFD2. These results highlight the importance of the ERGIC-53/MCFD2 interaction for the efficient secretion of coagulation factors V and VIII.

Capturing protein interactions in the secretory pathway of living cells

Numerous transient protein-protein interactions guide newly synthesized proteins through the secretory pathway. Most of these interactions are of low affinity and transient, and therefore difficult to study. We have developed a yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA) to detect protein-protein interactions in the secretory pathway of living cells. YFP fragments were fused to ERGIC-53, the ERGIC-53-interacting multi-coagulation factor deficiency protein MCFD2, and to ERGIC-53's cargo glycoprotein cathepsin Z. YFP PCA analysis revealed the oligomerization of ERGIC-53 and its interaction with MCFD2 (Figure 2), as well as its lectin-mediated interaction with cathepsin Z and cathepsin C. We conclude that YFP PCA can detect weak and transient protein interactions in the secretory pathway and hence is a powerful approach to study luminal processes involved in protein secretion, including carbohydrate-mediated protein-protein interactions of low affinity. We have now developed a YFP PCA-based genome-wide screening strategy that can be used to detect novel protein interactions in the lumen of the secretory pathway. This approach is currently used to find new cargo proteins of ERGIC-53.

Control of traffic in the secretory pathway by kinases and phosphatases

Kinases and phosphatases are key enzymes regulating diverse cellular processes. In collaboration with M. Zerial we have conducted a siRNA high throughput screen for human kinases and phosphatases involved in controlling traffic routes in the secretory pathway. This screen has yielded numerous hits that are currently studied in detail.

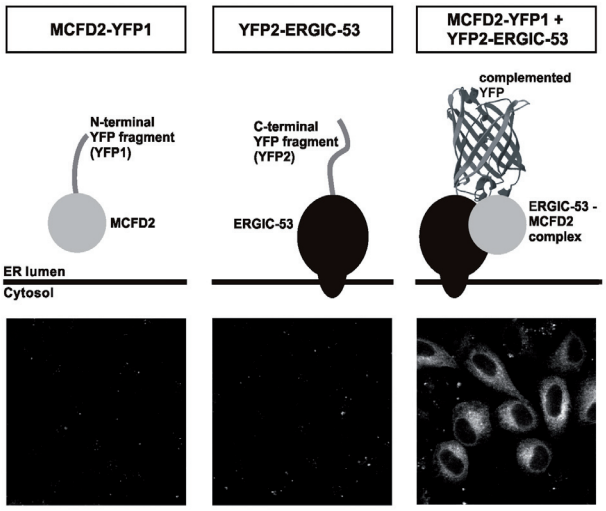

Fig. 2: Illustration of the YFP-PCA concept using the two interacting proteins MCFD2 and ERGIC-53 which were tagged with YFP1 and YFP2, respectively. Fluorescence microscopy of living HeLa cells (lower panel) shows no fluorescence if the two fusion proteins are expressed separately. Co-expression of MCFD2-YFP1 and YFP2-ERGIC-53, however, induces YFP fluorescence and thereby indicates interaction of ERGIC-53 and MCFD2 early in the secretory pathway.

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Dynamics of the cyclic di-GMP signaling network in development and cell cycle

Pathogenic and non-pathogenic bacteria can switch between a motile single cell state and a surface adherent community life-style called biofilms. Careful and efficient control of this transition is imperative for fitness and survival of microorganisms in structured environments. Our work aims at understanding the fundamental signaling principles and mechanisms underlying this general switch in microbial physiology. We study cellular dynamics in *Caulobacter crescentus*, an aquatic bacterium that has integrated the motile-sessile switch into its asymmetric replicative cycle. Our studies have proposed that the second messenger cyclic di-GMP is a key regulator of *Caulobacter* development and cell cycle progression. Cellular levels of c-di-GMP are controlled through the opposing activities of diguanylate cyclases (DGC) and phosphodiesterases (PDE), which represent two large families of output domains found in bacterial one- and two-component signal transduction systems. Using *Caulobacter* as model system we have pioneered some of the emerging concepts of c-di-GMP signaling in bacteria, including the original assignment of DGC and PDE activities to GGDEF and EAL domains, respectively; the first structural characterization of a diguanylate cyclase in its inactive and activated state; the identification of general allosteric mechanisms involved in synthesis and degradation of c-di-GMP; the molecular basis of activation and cellular dynamics of diguanylate cyclases; and the identification and functional characterization of distinct families of diguanylate receptor proteins. Progress in the past two years has been spurred by successful collaborations with groups in Structural Biology of the Biozentrum (T. Schrimer, S. Grzesiek) and the Chemistry Department (B. Giese, M. Meuwly).

Mechanisms of activation and cellular dynamics of the diguanylate cyclase PleD

Diguanylate cyclases are key signaling components in bacteria. Despite their importance and abundance in bacteria, catalytic and regulatory mechanisms of this enzyme class are poorly defined. To address this question we have performed *in vitro* and *in vivo* analyses of the *Caulobacter crescentus* diguanylate cyclase PleD. During the cell cycle PleD is activated by phosphorylation and sequestered to the differentiating cell pole where it orchestrates pole morphogenesis. We have shown that activation of PleD relies on a novel regulatory mechanism for cell fate determination, which involves posttranslational kinase feedback loops. Antagonistic activities of the PleC phosphatase and DivJ kinase localized at opposite cell poles control the phosphorylation

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state and subcellular localization of the cell fate determinator protein DivK. We have shown that DivK functions as an allosteric regulator that strongly stimulates both PleC and DivJ kinase activities and thereby mediates PleD-dependent execution of the stalked cell-specific morphogenetic program during G1-to-S transition. These data provide a logic framework for the cellular wiring of the network components that control asymmetry and offer a novel molecular mechanism for DivK as the central effector of the regulatory machinery that determines cell fate in *Caulobacter*. Based on these results we predict single domain response regulators to play key roles in the bacterial two-component signaling circuitry by providing the possibility for crosstalk, feedback control, and long-range communication in the cell.

We have shown that PleD can be specifically activated by beryllium fluoride *in vitro*, resulting in dimerization and c-di-GMP synthesis. Cross-linking and fractionation experiments were used to demonstrate that the dimer represents the enzymatically active form of PleD. In contrast to the catalytic activity, allosteric feedback regulation of PleD (see below) is not affected by the dimerization status of the protein, indicating that activation by dimerization and product inhibition represent independent layers of DGC control. The activation-by-dimerization mechanism received strong support from the crystal structure of beryllium fluoride activated PleD, which was solved by our collaborators in the Structural Biology unit. Finally, we could demonstrate that dimerization also serves to sequester activated PleD to the differentiating *Caulobacter* cell pole. This finding not only provides a molecular explanation for the coupling of PleD activation and subcellular localization but proposes protein oligomerization as general mechanism controlling cellular dynamics of proteins.

Allosteric feedback control of bacterial diguanylate cyclases

By using a combination of genetic, biochemical, and modeling techniques we have demonstrated that an allosteric binding site for c-di-GMP (I-site) is responsible for non-competitive product inhibition of DGCs. The I-site was mapped in multi- and single domain DGC proteins and is fully contained within the GGDEF domain itself. *In vivo* selection experiments and kinetic analysis of the evolved I-site mutants led to the definition of an RXXD motif as the core c-di-GMP binding site. Based on these results and based on the observation that the I-site is conserved in a majority of known and potential DGC proteins, we propose that product inhibition of DGCs is of fundamental importance for c-di-GMP signaling and cellular homeostasis by establishing precise threshold concentrations of the second messenger and by providing robustness to the regulatory network. The definition of the I-site binding pocket provides an entry point into unraveling the molecular mechanisms of ligand-protein interactions involved in c-di-GMP signaling, and makes DGCs a valuable target for drug design to develop new strategies against biofilm-related diseases.

Role of c-di-GMP in cell cycle progression

In *Caulobacter crescentus*, cell cycle progression relies on the periodic synthesis and degradation of the master cell cycle regulator CtrA, which is controlled by spatially sequestration of both substrate and protease. During the G1-to-S transition CtrA is transiently sequesters to the maturating old cell pole, where it is degraded by the polar localized ClpXP protease complex. We have identified PopA as pole specificity marker for CtrA sequestration and subsequent degradation. In *popA* mutants CtrA and the adaptor protein RcdA fail to sequester to the old cell pole and, as a consequence, CtrA remains stable upon entry into S-phase. PopA harbors a GGDEF output domain with a conserved allosteric binding pocket (I-site) and a degenerate active site motif GGDEF (A-site). Biochemical analysis showed that PopA lacks diguanylate cyclase but specifically binds c-di-GMP at the I-site with high affinity. Consistent with a role for PopA as a pole specificity factor, a functional PopA-GFP fusion protein dynamically localizes to the new cell pole during the *C. crescentus* cell cycle where it directly interacts with RcdA.

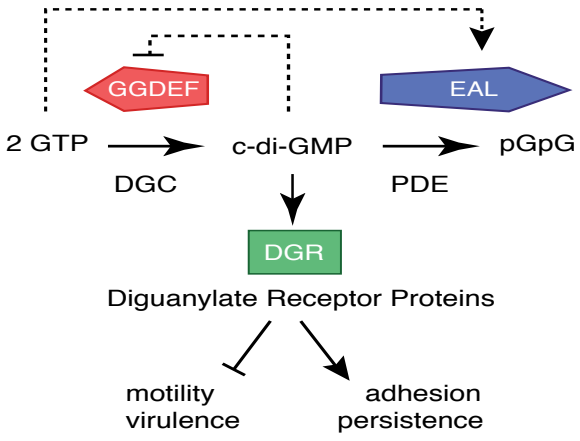

Fig. 1: Schematic of c-di-GMP synthesis, degradation and regulatory targets. DGC (diguanylate cyclase), PDE (phosphodiesterase); stippled lines indicate allosteric regulation of DGC and PDE activities.

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Based on the observation that PopA sequestration and CtrA degradation require a functional I-site, we propose a model in which PopA, in response to c-di-GMP binding, dynamically localizes to the maturating pole during the *C. crescentus* cell cycle, where it helps to recruit CtrA and possibly other substrates of the spatially sequestered ClpXP protease complex. This is the first example of a second messenger mediating spatio-temporal control of the bacterial cell cycle machinery.

DgrA, a member of a novel c-di-GMP effector family

Whereas the enzymatic mechanisms responsible for synthesis and breakdown of c-di-GMP have been elucidated, the downstream effector mechanisms have remained enigmatic. Using affinity chromatography we have isolated several c-di-GMP binding proteins from *Caulobacter crescentus*. One of these proteins, DgrA, is a member of the large PilZ domain protein family and is involved in mediating c-di-GMP-dependent control of *C. crescentus* cell motility. Biochemical and structural analysis of DgrA and homologs from *C. crescentus, Salmonella typhimurium* and *Pseudomonas aeruginosa* demonstrated that this protein family represents the first class of specific diguanylate receptors and suggested a general mechanism for c-di-GMP binding and signal transduction. Increased concentrations of c-di-GMP or DgrA blocked motility in *C. crescentus* by interfering with motor function rather than flagellar assembly. These studies represent an entry point into elucidating the molecular mechanisms underlying c-di-GMP-mediated cellular control.

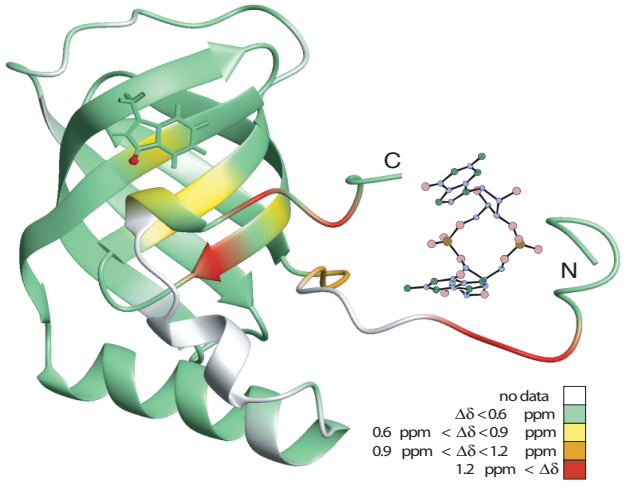

Fig. 2: Structure of the PilZ family protein PA4608 from P. aeruginosa. Combined amide 1H and 15N shift differences (Δδ̄values) between the free and ligand-bound form are indicated in color. One molecule of c-di-GMP is shown in the ball-and-stick model.

Principles of bacterial senescence

According to evolutionary theory, aging evolves because selection late in life is weak and mutations exist whose deleterious effects manifest only late in life. Whether the assumptions behind this theory are fulfilled in all organisms, and whether all organisms age, has not been clear. We tested the generality of this theory by experimental evolution with *Caulobacter crescentus*, a bacterium whose asymmetric division allows mother and daughter to be distinguished. We evolved three populations for 2000 generations in the laboratory under conditions where selection was strong early in life, but very weak later in life. All populations evolved significantly faster growth rates, mostly by decreasing the age at first division. Evolutionary changes in aging were inconsistent. The predominant response was the unexpected evolution of slower aging, revealing the limits of theoretical predictions if mutations have unanticipated phenotypic effects. However, we also observed the spread of a mutation causing earlier aging of mothers whose negative effect was reset in the daughters. These results confirm that late-acting deleterious mutations do occur in bacteria and that they can invade populations when selection late in life is weak. This suggests that very few organisms – perhaps none – can avoid the accumulation of such mutations over evolutionary time, and thus that aging is probably a fundamental property of all cellular organisms.

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Processing and editing of eukaryotic messenger RNA precursors and of transfer RNAs

The RNA processing reactions capping, splicing, 3‘ end cleavage/polyadenylation and RNA editing are important steps in the generation of nuclear messenger RNAs in eukaryotic cells. Alternative splicing and RNA editing give rise to alternate protein products that can have different functional properties. The cap at the 5’ end and the poly (A) tails at the 3’ end of mRNAs have multiple functions, such as increasing the efficiency of translation, mRNA export and the control of mRNA turnover. Moreover, the different processing steps are coupled to each other and to transcription by RNA polymerase II. Elucidation of the functional and structural properties of the factors involved in these reactions is the main focus of our research.

Mammalian pre-mRNA 3‘ end processing factors

3’ end formation is an essential step in the maturation of almost all primary RNA polymerase II transcripts that encode proteins. The 3’ end formation reaction includes endonucleolytic cleavage of the pre-mRNA, followed by polyadenylation of the upstream cleavage product. The addition of a poly(A) tail occurs cotranscriptionally and is dependent on functional interactions between the 3’ end formation machinery and RNA polymerase II. Six transacting factors are sufficient to catalyze mammalian 3’ end formation *in vitro*: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors I and II (CF I, CF II), poly(A) polymerase (PAP), and nuclear poly(A) binding protein (PABPN1). The functions of the large number of polypeptides constituting these factors include activities that are directly involved in the cleavage and polyadenylation steps and also in the coupling of this reaction to transcription and to other RNA processing reactions such as capping and splicing.

Because it proved impossible to purify all components of the mammalian 3’ end processing apparatus to homogeneity by conventional methods we attempted the heterologous expression of subcomplexes of the 3’ end processing apparatus in baculovirus-infected insect cells as well as the isolation of HeLa and HEK293 cells stably transformed with tagged subunits. We hope that by these approaches we can isolate different 3’ end processing factors from cell lysates by affinity purification via the tagged subunits. We have generated active CF I and CstF in the baculovirus system as well as by affinity purification from transformed cell lines. We have purified active CF II by affinity purification from a cell line pro-

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vided by Dr. Chris Trotta (PTC Therapeutics, South Plainfield, New Jersey). With the same strategy we have made stable cell lines expressing tagged subunits from which we could prepare CPSF by affinity purification. This CPSF preparation was specifically active in a reconstitution assay for polyadenylation.

We have identified subunits of the U2 snRNP in highly purified CPSF and showed that the two complexes physically interact. We therefore tested whether this interaction contributes to the coupling of 3' end processing and splicing. We found that CPSF is necessary for efficient splicing activity in coupled assays and that mutations in the pre-mRNA binding site of the U2 snRNP resulted in impaired splicing and in much reduced cleavage efficiency. Moreover, we showed that efficient cleavage required the presence of the U2 snRNA in coupled assays. Thus, we conclude that direct interactions between subunits of CPSF and the U2 snRNP contribute to the coupling of pre-mRNA 3' end processing and splicing.

Our longtime collaborator Dr. Sylvie Doublié has determined the crystal structure of the 25 kDa subunit of human cleavage factor I_m (CF I_m). CF I_m is an oligomer composed of a small 25kDa subunit (CF I_m25) and a larger subunit of either 59kDa, 68kDa, or 72kDa. The smaller subunit also interacts with RNA, poly(A) polymerase and PABPN1. These protein-protein interactions are thought to be facilitated by the Nudix domain of CF I_m25, a hydrolase motif with a characteristic α/β/α fold and a conserved catalytic sequence or Nudix box. We determined the crystal structures of human CF I_m25 in its free and diadenosine tetraphosphate (Ap4A) bound forms at 1.85Å and 1.80Å, respectively. CF I_m25 crystallizes as a dimer in both structures and presents the classical Nudix fold. Results from crystallographic and biochemical experiments suggest that CF I_m25 makes use of its Nudix fold to bind but not hydrolyze the nucleotide substrates ATP and Ap4A. The binary complex also reveals, unlike previously characterized Nudix enzymes, that CF I_m25 uses residues outside of the Nudix box for nucleotide binding. The complex and apo protein structures provide insight into the active oligomeric state of CF I_m and suggest a possible role of nucleotide binding in either the polyadenylation and/or cleavage steps of pre-messenger RNA 3' end processing.

3' end processing of mRNA precursors in yeast

3' end processing of yeast pre-mRNAs *in vitro* requires cleavage factor IA (CF IA), cleavage factor IB (CF IB) and cleavage and polyadenylation factor (CPF). CF IA consists of four subunits, CF IB is a single polypeptide and CPF is a multiprotein complex of about 15 polypeptides.

The yeast cleavage and polyadenylation factor (CPF) is a multiprotein complex that plays a key role in pre-mRNA 3' end processing. To understand the arrangement of the subunits in the CPF complex, we have developed a new method that allows the isolation of highly purified CPF. In collaboration with Dr. Holger Stark and the group of Professor Andreas Engel we have determined the three-dimensional structure of the complex at a resolution of 25Å. This structure was obtained by negatively stained electron microscopy (EM) preparations of CPF, angular reconstitution and random conical tilt. After two affinity purification steps, homogenous CPF populations suitable for EM analysis were obtained by combining density and cross-linker gradients in the same centrifuge tube. Our model reveals that CPF has a complex asymmetric architecture in which an outer protein wall surrounds a large inner cavity. We are currently using cryo-electron microscopy (cryo-EM) to improve the resolution of this model and to localize subunits by fitting available X-ray structures or GFP tagged proteins. We have also used scanning transmission electron microscopy (STEM) to determine the molecular mass of CPF, which we have found to be close to one megadalton, consistent with the predicted mass obtained by the sum of the masses of the individual subunits. This suggests a one to one relative stoichiometry of subunits within the complex.

Non-canonical poly(A) polymerases

As reported earlier, we have characterized a new type of protein complex with poly(A) polymerase activity from *Saccharomyces cerevisiae* that targets RNAs destined for degradation and functions as an activator of the nuclear exosome. Eukaryotic cells contain several unconventional poly(A) polymerases in addition to the canonical enzymes responsible for the synthesis of poly(A) tails of nuclear messenger RNA precursors. We continued to characterize the biochemistry of the yeast Trf4 complex. One of the important open questions is how the Trf4-complex binds it target RNAs and how it can distinguish correctly folded from aberrant RNA molecules. The catalytic subunit of the Trf4 complex has been previously cloned and expressed from the pET22b vector, however the expression levels and solubility were poor. To optimize the expression of recombinant Trf4 (rTrf4), a fusion of Trf4p and G-protein has been subcloned into another vector, which provided high lev-

els of expression of soluble protein in *E. coli*. Similar fusion proteins have been prepared for Air2p and Air1p. One of the aims is to solve the structure of single subunits of the Trf4/Air complex. To further scrutinize the RNA and rTrf4 binding properties of the Air1 and Air2 proteins, constructs with deletion versions of the Air proteins have been prepared. The deletion forms include plasmids encoding the five-zinc knuckle domain alone or with the N- or C-terminus. The expressed and purified proteins will be tested for tRNA-binding by gel-shift assays and the protein-protein interactions are being analyzed by GST- and His-tag pulldown experiments. The interactions with different tRNA targets is being studied by chemical and enzymatic structure probing.

RNA-specific adenosine deaminases and RNA editing

RNA-specific adenosine deaminases which convert adenosine to inosine residues are divided into two families: adenosine deaminases acting on RNA (ADARs) act on double-stranded RNA and edit several cellular pre-mRNAs whereas ADATs convert adenosine to inosine in tRNAs. *E. coli* TadA has been the first prokaryotic ADAT identified and is essential for cell viability. TadA exhibits high sequence similarity to the yeast tRNA deaminase subunit Tad2p and converts the adenosine at the wobble position 34 of $tRNA^{Arg2}$ into inosine. Editing at the wobble position is predicted to enable $tRNA^{Arg2}$ to decode the arginine codons CGU, CGA and CGC, thus increasing protein translation efficiency *in vivo*. We are testing this prediction experimentally. We are also planning to identify the location of TadA in the cell and to investigate whether in addition to its role in $tRNA^{Arg2}$ editing TadA can act on other RNA substrates.

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Networks controlling polar growth and nuclear dynamics in fungi

Introduction

We investigate differences and similarities in cellular networks of two eukaryotic microorganisms, the unicellular fungus *Saccharomyces cerevisiae* and the filamentous fungus *Ashbya gossypii*. Despite their differences in life style and growth habitat both organisms evolved from a common ancestor appr. 100 Million years ago (Dietrich *et a*l. 2004, *Science*). Two key functions, polar growth and nuclear dynamics, are controlled in both organisms by similar sets of genes. The majority of these genes are present at syntenic positions (conserved gene order) confirming their evolution from common ancestral genes. During evolution, the individual role of many of these genes and the interaction network as a whole was modified to produce the differences in growth and nuclear dynamics observed today. Growth of *A. gossypii* is restricted to the tip of the long multinucleated cells, called hyphae. Once established, this leads to continous hyphal elongation and branching forming a typical fungal mycelium. The surface expansion rate of *A. gossypii* hyphae is 20 to 40 times faster compared to the growth rate of *S. cerevisiae* cells. We hypothesize that components controlling the actin cytoskeleton and/or exocytosis, endocytosis as well as cell wall synthesis have evolved differently in both organisms. Homologs to all *S. cerevisiae* genes known to control nuclear movements and divisions are encoded in *A. gossypii*. These homologs are assumed to control nuclear oscillation, bypassing, and division in multinucleated hyphae. One of the most remarkable differences between both organisms is the lack of cell separation after nuclear divisions in *A. gossypii*. We hypothesize that novel modes of regulation of conserved cell cycle proteins may direct nuclear dynamics in this fungus.

The SH3 protein AgBoi1/2 collaborates with AgRho3 to prevent nonpolar growth phases at hyphal tips of *Ashbya gossypii*

Unlike most other cells, hyphae of filamentous fungi permanently elongate and lack nonpolar growth phases. We identified AgBoi1/2p in the filamentous ascomycete *Ashbya gossypii* as a component required to prevent nonpolar growth at hyphal tips. Strains lacking AgBoi1/2p frequently show spherical enlargement at hyphal tips with concomitant depolarization of actin patches and loss of tip-located actin cables. These enlarged tips can repolarize and resume hyphal tip extension in the previous polarity axis. AgBoi1/2p permanently localizes to hyphal tips and transiently to sites of septation. Only the tip localization is important for sustained elongation of

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hyphae. In a yeast two-hybrid experiment, we identified the Rho-type GTPase AgRho3p as an interactor of AgBoi1/2p. AgRho3p is also required to prevent nonpolar growth at hyphal tips, and strains deleted for both Ag*BOI1/2* and Ag*RHO3* phenocopied the respective single-deletion strains, demonstrating that AgBoi1/2p and AgRho3p function in a common pathway. Monitoring the polarisome of growing hyphae using AgSpa2p fused to the green fluorescent protein as a marker, we found that polarisome disassembly precedes the onset of nonpolar growth in strains lacking AgBoi1/2p or AgRho3p. AgRho3p locked in its GTP-bound form interacts with the Rho-binding domain of the polarisome-associated formin AgBni1p, implying that AgRho3p has the capacity to directly activate formin-driven actin cable nucleation. We conclude that AgBoi1/2p and AgRho3p support polarisome-mediated actin cable formation at hyphal tips, thereby ensuring permanent polar tip growth.

A novel role of a formin in morphogenesis of *Ashbya gossypii*

Morphogenesis of filamentous ascomycetes includes continuously elongating hyphae, frequently emerging lateral branches, and, under certain circumstances, symmetrically dividing hyphal tips. We identified the formin *Ag*Bni1p of the model fungus *Ashbya gossypii* as an essential factor in these processes. *Ag*Bni1p is an essential protein apparently lacking functional overlaps with the two additional *A. gossypii* formins that are nonessential. *Agbni1* null mutants fail to develop hyphae and instead expand to potato-shaped giant cells, which lack actin cables and thus tip-directed transport of secretory vesicles. Consistent with the essential role in hyphal development, AgBni1p locates to tips, but not to septa. The presence of a diaphanous autoregulatory domain (DAD) indicates that the activation of *Ag*Bni1p depends on Rho-type GTPases. Deletion of this domain, which should render *Ag*Bni1p constitutively active, completely changes the branching pattern of young hyphae. New axes of polarity are no longer established subapically (lateral branching) but by symmetric divisions of hyphal tips (tip splitting). In wild-type hyphae, tip splitting is induced much later and only at much higher elongation speed. When GTP-locked Rho-type GTPases were tested, only the young hyphae with mutated *Ag*Cdc42p split at their tips, similar to the DAD deletion mutant. Two-hybrid experiments confirmed that *Ag*Bni1p interacts with GTP-bound *Ag*Cdc42p. These data suggest a pathway for transforming one axis into two new axes of polar growth, in which an increased activation of *Ag*Bni1p by a pulse of activated *Ag*Cdc42p stimulates additional actin cable formation and tip-directed vesicle transport, thus enlarging and ultimately splitting the polarity site.

AgPxl1 is required for symmetric tip splitting

The development from young, slowly growing hyphae to fast growing hyphae in filamentous fungi is referred to as hyphal maturation. One landmark of maturation is the symmetric division at the tips of fast growing hyphae simultaneously generating two sister hyphae. We have identified the Paxillin-like protein AgPxl1 as the first protein that is specifically required for hyphal tip splitting. This event is mechanistically different from the well known lateral branching during early hyphal maturation. The early development of *A. gossypii* strains lacking AgPxl1 is indistinguishable from wild type as judged from acceleration of hyphal extensions, branching indices, and branching frequencies. However, two thirds of the tip splitting events lead to sister hyphae either elongating with different speeds or to sister hyphae with an immediate growth arrest of one hypha. In the latter cases the polarisome still divides but disassembles at the non-growing tip indicating a direct or indirect role of AgPxl1 in stabilizing the polarisome at high elongation speeds. This may also explain the about 60% decreased maximal elongation speed of hyphae lacking AgPxl1. The conserved carboxy-terminal LIM domains are necessary for AgPxl1 function and also contribute to the tip localisation. AgCLA4, a PAK-like kinase is epistatic to AgPXL1 because single and double deletions display the same tip splitting defects. In addition, the robust localisation of AgPxl1 depends on AgCla4.

The function of two related Rho proteins is determined by an atypical switch I region

The majority of small Rho-type GTP/GDP-binding proteins present in cells of higher organisms evolved by gene duplications. While the core switch mechanism of these proteins remained strongly conserved, their interface with interacting proteins was often altered leading to differentiation of function. We found a novel functional change after duplication of the *RHO1* gene from *A. gossypii*. The encoded proteins, *Ag*Rho1a and *Ag*Rho1b, functionally diverged in their switch I region. Interaction studies and *in vitro* assays suggest that additionally a modified regulation by the two GTPase activating proteins (GAP) *Ag*Lrg1 and *Ag*Sac7 contributes to those differences. GAP-specificity and protein function are determined to a large part by a single position in the switch I region of the two Rho1 proteins. In *Ag*Rho1b this residue is a

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tyrosine, which is conserved among the Rho-protein family, while *Ag*Rho1a carries an atypical histidine at the same position. Mutation of this histidine to a tyrosine changes GAP-specificity and protein function of *Ag*Rho1a and enables the mutated allele to complement the lethality of an *Ag*RHO1b deletion.

Dynamics of the actin cytoskeleton in *A. gossypii*

Filamentous fungi develop from a spore to a network of permanently elongating and branching multinucleated cells, called hyphae. Surface expansion of hyphae is restricted to their tips which, depending on the species, can result in hyphal elongation speeds of several micrometers per minute or more. Essential components of cell surface growth are actin patches and cables and associated type I and type V myosins. We used the model fungus *Ashbya gossypii* to monitor dynamic properties of actin-based structures and motors in strictly polarly growing cells (elongation speed 0.4-0.8 µm/min, diameter 4 µm). AgCap1-GFP or AgCap2-GFP labeled actin patches have a life time of 10 to 25 seconds. They form with high density mainly at the rim of the hyphal tip, reside for a few seconds, and then move retrograde with speeds of 100 to 400 nm/sec. 15% of the actin patches recycle to the tip. *A. gossypii* codes for a single type I myosin. A carboxy-terminal GFP-fusion of this myosin forms small fluorescent dots mainly at the hyphal tip with a life time of only 3 to 6 seconds, indicating very efficient fission of endocytic vesicles. Hyphae lacking this myosin grow very slowly with irregular diameters and actin patches are no longer concentrated at the tip region but are distributed along the hyphal cortex. Two to three AgAbp140-GFP labeled actin cables emanate from hyphal tips with elongation speeds of 100 to 300 nm/sec very similar to the retrograde migration of actin patches. Time-lapse movies of AgSec4-GFP labeled secretory vesicles revealed transport speeds of 1000 nm/sec and more concomitant with an accumulation of fluorescence at the tip. Motors responsible for vesicle transport are type V myosins. Deletion of the single *A. gossypii* gene coding for a type V myosin prevents polar growth, instead the surface of germinating mutant spores isotropically expands for many hours showing high density of cortical actin patches but collapsed bundles of actin cables. Using a carboxy-terminal GFP-fusion of this myosin we measured tip-directed migration speeds of 1000 nm/sec and more. The resulting high accumulation of dynamic fluorescent clouds at the tip indicates a very dense population of vesicles transported along actin cables to the polar growth zone of hyphae.

***A. gossypii* homologues of *S. cerevisiae* BUD genes**

A. gossypii carries homologues of *ScRAX1*, *ScRAX2*, *ScBUD7*, and the twin genes *ScBUD8, ScBUD9*. These genes encode putative transmembrane proteins. We are interested in determining the role of these homologues in the filamentous growth pattern of *A. gossypii*. In contrast to *A. gossypii* wild type, in Agrax1, Agrax2 and Agbud8/9 deletions up to four branches can simultaneously emerge from the main hypha, and additional new branches are formed between the already developed ones. Tip growth of the main hypha is markedly reduced when branches start growing. An altered branching pattern was not observed in the *Agbud7* deletion. AgRax2-GFP localizes at the tips of hypha and at the septum where it appears just after actomyosin ring formation, and before chitin deposition. AgBud7-GFP is found in dot-like structures which oscillate. The observed structures are able to divide keeping a similar density throughout the hyphae.

Limited functional redundancy and oscillation of cyclin abundance in *Ashbya gossypii*

Cyclin protein behavior has not been systematically investigated in multinucleated cells like fungal hyphae. Cyclins are canonical oscillating cell cycle proteins, but it is unclear how fluctuating protein gradients can be established in multinucleated cells where nuclei in different stages of the division cycle share the cytoplasm. Previous work in *A. gossypii*, a filamentous fungus in which nuclei divide asynchronously in a common cytoplasm, demonstrated that one G1 and one B-type cyclin do not fluctuate in abundance across the division cycle. We have undertaken a comprehensive analysis of all G1 and B-type cyclins in *A. gossypii* to determine whether any of the cyclins show periodic abundance across the cell cycle and to examine whether cyclins exhibit functional redundancy (as previously demonstrated in *S. cerevisiae*) in such a cellular environment. We localized all G1 and B-type cyclins and notably found that only AgClb5/6p varies in subcellular localization during the division cycle. AgClb5/6p is lost from nuclei at the meta-anaphase transition in a D-box-dependent manner. These data demonstrate that efficient nuclear autonomous protein degradation can occur within multinucleated cells residing in a common cytoplasm. We have shown that three of the five cyclins in *A. gossypii* are essential genes, indicating that there is minimal functional redundancy in this multinucleated system. In addition, we have identified a cyclin, AgClb3/4p, that is essential only

for sporulation. We propose that the cohabitation of different cyclins in nuclei has led to enhanced substrate specificity and limited functional redundancy within classes of cyclins in multinucleated cells.

AgSwe1p regulates mitosis in response to morphogenesis and nutrients in *Ashbya gossypii*

Nuclei in *A. gossypii* hyphae divide asynchronously. We have investigated what internal and external signals spatially direct mitosis within these multinucleated cells. Mitoses are most common near cortical septin rings found at growing tips and branch points. In septin mutants, mitoses are no longer concentrated at branch points suggesting that the septin rings function to locally promote mitosis near new branches. Similarly, cells lacking AgSwe1p kinase (a Wee1 homologue), AgHsl1p (a Nim1-related kinase) and AgMih1p phosphatase (the Cdc25 homologue that likely counteracts AgSwe1p activity) also have mitoses distributed randomly in the hyphae as opposed to at branch points. Surprisingly, however, no phosphorylation of the CDK tyrosine 18 residue, the conserved substrate of Swe1p kinases, was detected in normally growing cells. In contrast, abundant CDK tyrosine phosphorylation was apparent in starving cells resulting in diminished nuclear density. This starvation-induced CDK phosphorylation is AgSwe1p dependent and overexpressed AgSwe1p is sufficient to delay nuclei even in rich nutrient conditions. In starving cells lacking septins or AgSwe1p negative regulators, the nuclear density is further diminished compared with wild type. We have generated a model in which AgSwe1p may regulate mitosis in response to cell intrinsic morphogenesis cues and external nutrient availability in multinucleated cells.

Anaphase progression in multinucleated *Ashbya gossypii* cells

Regulated protein degradation is essential for eukaryotic cell cycle progression. The anaphase-promoting complex/cyclosome (APC/C) is responsible for the protein destruction required for the initiation of anaphase and the exit from mitosis, including the degradation of securin and B-type cyclins. We initiated a study of the APC/C in the multinucleated, filamentous ascomycete *Ashbya gossypii* to understand the mechanisms underlying the asynchronous mitosis observed in these cells. These experiments were motivated by previous work which demonstrated that the mitotic cyclin AgClb1/2p persists through anaphase, suggesting that the APC/C may not be required for the division cycle in *A. gossypii*. We have now found that the predicted APC/C components AgCdc23p and AgDoc1p and the targeting factors AgCdc20p and AgCdh1p are essential for growth and nuclear division. Mutants lacking any of these factors arrest as germlings with nuclei blocked in mitosis. A likely substrate of the APC/C is the securin homologue AgPds1p, which is present in all nuclei in hyphae except those in anaphase. The destruction box sequence of AgPds1p is required for this timed disappearance. To investigate how the APC/C may function to degrade AgPds1p in only the subset of anaphase nuclei, we localized components and targeting subunits of the APC/C. Remarkably, AgCdc23p, AgDoc1p, and AgCdc16p were found in all nuclei in all cell cycle stages, as were the APC/C targeting factors AgCdc20p and AgCdh1p. These data suggest that the AgAPC/C may be constitutively active across the cell cycle and that proteolysis in these multinucleated cells may be regulated at the level of substrates rather than by the APC/C itself.

Septins in *A. gossypii*

In *S. cerevisiae*, the septins are thought to link bud formation to mitosis by recruiting Hsl1p and Hsl7p, which are required for Swe1p inactivation and entry into mitosis. The proper assembly and organization of the septin collar at the neck is essential for the function of these cell cycle regulators. In *A. gossypii*, homologues to all key players of this morphogenesis checkpoint exist. To begin to investigate possible connections between the cell cycle and morphogenesis we have focused on analyzing the structure and function of septin proteins in *A. gossypii* by using a Sep7-GFP strain. We detected some septin structures that were not described so far. Interestingly, most of the structures in *A. gossypii* seem to be composed of parallel bars, an instance which in yeast is only seen in certain mutants and is associated with higher Swe1p kinase activity. Heterologous expression of AgSep7-GFP in *S. cerevisiae* leads to the formation of normal yeast structures with continuous rings and double rings. This implies that the observed structures are not inherent to the Sep7 protein, but rather the result of different regulation or interaction in the two organisms.

Ashbya Genome Database 3.0

The Ashbya Genome Database (AGD) 3.0 is an innovative cross-species genome and transcriptome browser based on release 40 of the Ensembl developer environment. AGD 3.0 provides information on 4726 protein-encoding loci and 293 non-coding RNA genes present in the genome of the filamentous fun-

gus Ashbya gossypii. A synteny viewer depicts the chromosomal location and orientation of orthologous genes in the budding yeast *Saccharomyces cerevisiae*. Genome-wide expression profiling data obtained with high-density oligonucleotide microarrays (GeneChips) are available for nearly all currently annotated protein-coding loci in *A. gossypii* and *S. cerevisiae*. AGD 3.0 hence provides yeast- and genome biologists with comprehensive report pages including reliable DNA annotation, Gene Ontology terms associated with *S. cerevisiae* orthologues and RNA expression data as well as numerous links to external sources of information. The database is accessible at http://agd.vital-it.ch/.

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Intracellular transport in yeast and worms

Intracellular transport is essential for the survival of cells. The different organelles within a cell communicate with each other and exchange lipids and proteins primarily via coated vesicles. The generation of these vesicles must be tightly regulated in order to maintain the integrity of the different organelles including the plasma membrane. However, the different organelles are also not just static entities distributed throughout the cytoplasm of the cell; they are dynamic as their shape can change in response to the cell cycle, or their abundance or size can be controlled in response to stress or other signals. Furthermore, organellar structures are often compartmentalized, and this compartmentalization needs to be maintained. Classical examples are the exit sites at the endoplasmic reticulum (ER), the rough and smooth ER and different parts of the plasma membrane. Interestingly, the communication between organelles depends on small GTPases of the ras superfamily. One class of small GTPases – the family of Arf and Sar GTPases – is essential for the generation of transport carriers, while another class – the rab GTPases – is involved in the consumption of transport carriers and seems to play an essential role in the maintenance of organellar identity. Our research interests center around the communication between and the dynamics of organelles in the cell, and the regulation of intracellular processes by small GTPases of the Arf and rab families.

We use the unicellular yeast *Saccharomyces cerevisiae* and the roundworm *Caenorhabditis elegans* for our studies as both organisms are particularly well suited to answer the kind of questions we like to address.

Regulation of intracellular transport

To study intracellular transport and the life cycle of a transport vesicle, we established *in vitro* systems that allow us to recapitulate transport in the ER-Golgi shuttle and vesicle formation from the ER and the Golgi in yeast or from synthetic, chemically defined liposomes (Spang *et al*., 1998, Spang and Schekman, 1998). These assays are extremely useful in the dissection of the function of transport pathways. For example, they enabled us to identify Ypt1p as the rab protein essential for the fusion of transport vesicles at both the Golgi apparatus and the ER. Furthermore, interference with Ypt1p functions gave rise leads to the accumulation of early Golgi marker in the ER and the dispersal of at least one medial Golgi enzyme, Anp1p, and the trans-Golgi associated Sec7p (Kamena *et al*. in revision). We are not only interested in the consumption of transport car-

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rier, but also how they are generated and how this process is controlled. To this end, we established roles for the effectors of the small GTPase Arf1p, ArfGAP (Arf1p GTPase activating protein) and Arf-GEF (Arf1p guanine nucleotide exchange factors), in COPI vesicle formation (Poon *et al*., 1999, Spang *et al*., 2001, Rein *et al*., 2002, Lewis *et al*., 2004, Robinson *et al*., 2006). Although at first it may appear counter-intuitive, but the negative regulator of Arf1p, the ArfGAP Glo3p, positively influences vesicle generation and is also required for the inclusion SNARE proteins into COPI vesicles. Glo3p is able to induce a conformational change in SNAREs, which allows their subsequent interaction with Arf1p and the coat complex coatomer. Interestingly, the fusion factor Sec18p/NSF could displace Arf1p from SNAREs, which may provide a mechanism by which residual coat proteins are removed from the vesicle and hence SNARE complex formation could proceed, which would then lead to fusion of the vesicle with the target membrane (Schindler and Spang, 2007). The cargo, which needs to be included into the vesicles is not just a passive bystander, but plays an active role. Overexpression of cargo proteins with a coatomer-binding sequence (-KKXX) could rescue coatomer mutants in the -KKXX recognizing subunit (Sandmann *et al*., 2003). Furthermore, we could recently show in collaboration with Manuel Muñiz that in the absence of the ArfGAP Glo3p, the p24 family proteins, which cycle between the ER and the Golgi apparatus, are required to bud vesicles efficiently from the Golgi (Aguilera *et al., in press*). Moreover, in collaboration with the group of Blanche Schwappach, we identified a novel bi-partite cargo recognition motif in coatomer (Michelsen *et al*. 2007). These results suggest that cargo-coat interaction stabilize the priming complexes proposed by Springer et al. (1999) and that the formation of coat-cargo complexes is an essential integral part of vesicle biogenesis. Despite all the knowledge we have gained to date about vesicle formation, we still do not know exactly when the coat is destabilized or shed. Experimental evidence is available for three possible scenarios: Firstly, during the budding process itself. The coat would disassemble at the tip of the emerging bud, while at the base coat polymerization still could take place. Secondly, after the budding process is completed, en route to the target compartment. And finally the coat could be required for the initial recognition process between vesicles and target compartment upstream of the SNAREs and would only be shed after the successful recognition. To distinguish between these possibilities is one of the problems we try to tackle currently.

Multiple transport pathways to the plasma membrane have been described in yeast. However, not for all the transport carriers the proteinaceous coat was known. We identified the ChAPs, Chs5p and Arf1p as being essential for the transport of the chitin synthase Chs3p to the plasma membrane, more precisely to the bud neck (Trautwein *et al*., 2006). We could show, that during a precise time frame in the cell-cycle Chs3p is not transported with the bulk of the other secreted or plasma membrane proteins. This finding suggests that there are specialized vesicles that transport only a subset of cargo proteins to defined place at the plasma membrane. This scenario is certainly conserved in higher eukaryotes, even though the specific transport machinery could be different. We are currently in the process of identifying more cargo proteins that take the same route as Chs3p to the bud neck. It is very likely that the transport of Chs3p from the trans-Golgi to the plasma membrane is regulated in a different manner than the export of cargo that is not confined to the bud neck. Candidates, which may regulate this special transport event, are currently under investigation.

Regulation of membrane dynamics

We are also interested in membrane dynamics and organelle structure and function. Studying the phosphorylation of the Golgi t-SNARE, Sed5p, we showed that phosphorylation of Sed5p prevents stacking of Golgi cisternae in *S. cerevisiae* (Weinberger *et al*. 2005). We could also show that the ER in *S. cerevisiae* has diffusion barrier at the bud neck, which is under control of septins (Luedecke *et al*., 2005). While no major rearrangements of ER membranes during the cell-cycle was observed in *S. cerevisiae*, in early *C. elegans* embryos, the ER undergoes dramatic changes upon entry into mitosis: sheets of ER are formed (Poteryaev *et al*., 2005). These sheets disperse into more reticulate structures just prior to cytokinesis. Hence, the ER cycles in *C. elegans* embryos between a reticulate and a sheet state. These cell-cycle-dependent changes involve Arf-1 and actin. We are currently trying to elucidate the mechanisms, which govern these dramatic changes and to understand the individual function of the different ER states. In our quest to understand membrane dynamics, we cloned a new effector of the small GTPase RAB-7, called SAND-1, which is essential for early to late endosomal transport in *C. elegans* (Poteryaev and Spang, 2005, Poteryaev *et al*., 2007). Moreover, loss-of-function of SAND-1 resulted in a dramatic increase of the early endosomal RAB-5 and of RAB-7 in the cell. In contrast, the recycling endosome-associated RAB-11 was unaffected under these condi-

tions. SAND-1 is a highly conserved protein, which may perform similar functions in mammalian cells. At present we are aiming to understand how SAND-1 influences RAB-5 and RAB-7 function. Since GTPases of the RHO family are particularly important for plasma membrane dynamics, usually in connection with actin, we characterized two novel RhoGAPs, RGA-3 and RGA-4 in the early *C. elegans* embryo and established an essential role in the modulation of RHO-1 activity and plasma membrane contractility (Schmutz *et al*. 2007). Interestingly, polarity establishment and maintenance were unaffected by the loss of RGA-3/4 function. RGA-3/4 function is not restricted to the early embryo. They are also required for germline development in worms when signaling of RHO-1 through the RHO-associated kinase LET-502 is impaired.

The above presented highlights only a part of the ongoing research efforts in the laboratory. More projects in the lab center around the question of the regulation of vesicle flow between different organelles (including the plasma membrane) in the cell. Our recent results led us to ask how vesicular transport and polarity establishment are connected in different systems. Furthermore, we are still uncovering novel roles for the small GTPase Arf1p and we are trying to understand how Arf1p is regulated performing these distinct function. The seminal work by the labs of Schekman and Rothman in the 80ies opened the field of membrane traffic. Almost 30 years later, lots of exciting and interesting questions still remain unanswered.

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Membrane protein topogenesis and intracellular protein sorting

Proteins synthesized on cytosolic ribosomes must be sorted to the specific compartment(s) in which they perform their function. Proteins of the endoplasmic reticulum (ER), the Golgi apparatus, the plasma membrane, the endosomal/lysosomal system and the cell exterior are all first targeted to the ER, translocated across the membrane or inserted into the lipid bilayer, and then distributed via the secretory pathway. Our research focuses on (1) how membrane proteins are inserted into the ER membrane and acquire a defined topology, and (2) how transport vesicles are formed at the trans-Golgi or endosomes, or in *vitro* from purified components and liposomes. In close collaboration with Jonas Rutishauser, we furthermore study the mechanism by which trafficking mutants of provasopressin cause dominant neurohypophyseal *diabetes insipidus*.

Topogenesis of membrane proteins

Proteins are targeted by hydrophobic signal sequences to the ER membrane where the Sec61/SecY translocon mediates the passage of hydrophilic sequences across the membrane and the integration of apolar transmembrane segments into the lipid bilayer. In this process, the topology of the polypeptide chain, i.e. the orientation of the signal and subsequent transmembrane segments in the membrane, is determined. We are studying the determinants and the process of topogenesis *in vivo* by challenging eukaryotic cells with diagnostic mutant proteins. Signal orientation is determined by the flanking charges, hydrophobicity, and folding of N-terminal sequences. We found that with very hydrophobic N-terminal signals, the process of signal orientation to an N_{cyt}/C_{exo} topology is dependent on the length of the protein, corresponding to the time of translation, both in mammalian cells and in yeast. This process may reflect the opening of the translocon for translocation of the C-terminal sequence.

The yeast system provides the possibility to study the effect of mutations in the translocon on topogenesis and on co- and post-translational translocation in general. The structure of the *Methanococcus* SecY revealed a plug domain blocking the pore on the lumenal side and a hydrophobic constriction ring that appeared to be important for gating the protein conducting channel and to maintain the permeability barrier in the unoccupied state. We analyzed in yeast the effect of destabilizing point mutations in the plug domain or of its partial or complete deletion. Unexpectedly, even when the entire plug domain was deleted, cells were viable without growth phenotype. They showed an effect on signal sequence orienta-

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tion of diagnostic signal-anchor proteins, a minor defect in co-translational and a significant deficiency in post-translational translocation. The results suggest that the plug is unlikely to be important for sealing the translocation pore in yeast, but that it plays a role in stabilizing Sec61p during translocon formation.

The orientation of most single-spanning membrane proteins obeys the „positive-inside rule“, i.e. the flanking region of the transmembrane segment that is more positively charged remains in the cytosol. We have screened for mutations in yeast Sec61p that alter the orientation of single-spanning membrane proteins. We identified a class of mutants that are less efficient in retaining the positively charged flanking region in the cytosol. Surprisingly, these mutations are located at many different sites in the Sec61/SecY molecule, and they do not only involve charged amino acid residues. All these mutants, as well as the plug deletion, have a *prl* phenotype that so far had only been seen in bacteria; they allow proteins with defective signal sequences to be translocated, likely because the Sec61p channel opens more easily. A similar correlation between topology defects and *prl* phenotype was also seen with previously identified yeast Sec61 mutants. Our results suggest a model in which the regulated opening of the translocon is required for the faithful orientation of membrane proteins.

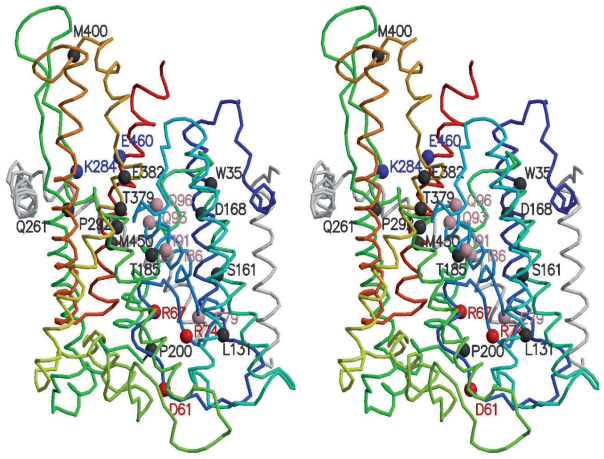

Fig. 1: Stereoimage of the yeast Sec61 complex protein backbone. Indicated are residues affecting protein topogenesis when mutated.

Formation of transport vesicles

Transport between organelles is generally mediated by cytosolic coats which serve to create a scaffold on the membrane to form coated buds and vesicles, and to selectively concentrate cargo by interacting with cytosolic signals. Clathrin coats with different adaptor proteins (APs) are involved in transporting cargo proteins with characteristic tyrosine and dileucine motifs in various intracellular pathways. We have developed an assay using permeabilized cells to study the molecular requirements for the recycling of internalized receptors back to the plasma membrane. Manipulation of the cytosol used for reconstitution revealed that formation of recycling vesicles requires AP-1/clathrin coats and is regulated by rab4 and the connector rabaptin-5. Cytosol of cultured cell lines may be used in the assay. This allows to easily test the effect of overexpressed proteins or dominant mutants.

The assay has also been adapted to analyze formation of transport carriers at the *trans*-Golgi network. To specifically label proteins in the trans-Golgi, we use [35S]sulfation. To introduce sulfation sites, proteins were tagged with short sequences containing sites for tyrosine sulfation or for glycosaminoglycan (GAG) attachment. Interestingly, the latter was found to affect protein transport by inhibiting endocytosis and by altering the mechanism of *trans*-Golgi exit.

To study the minimal mechanisms of coat formation, we reconstitute the recruitment of purified coat components to chemically defined liposomes. In particular, the effect of cargo is analyzed by coupling short peptides to the liposomes corresponding to functional or mutant sorting signals. Sorting peptides were fond to be necessary for the recruitement of AP-1 adaptors with Arf1•GTP and to cause AP-1 oligomerization even in the absence of clathrin. Interaction with cargo reduces the rate of ArfGAP1-induced GTP hydrolysis, thus contributing to efficient cargo sorting. In contrast, cargo signals of the COPII coat did not affect GTP hydrolysis of the GTPase Sar1p, but nevertheless stabilized recruitment of the inner layer of the coat (Sec23/24p) or of the full coat (including Sec13/31p). Cargo signals thus render the coat independent of Sar1p and GTP hydrolysis and thus promote productive vesicle formation.

Provasopressin: mutants vs. wild-type

Autosomal dominant neurohypophyseal *diabetes insipidus* results from mutations in the precursor protein of arginine vasopressin, the hormone regulating water reabsorption in the kidney. Mutant prohormone is retained in the ER of vasopressinergic neurons and causes them to undergo progressive degeneration, but the mechanisms of neurotoxicity are unknown. We discovered that dominant pro-va-

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sopressin mutants form disulfide-linked homo-oligomers and develop large aggregations visible by fluorescence and immunogold electron microscopy, both in a fibroblast and a neuronal cell line, frequently with a fibrillar substructure. These findings assign autosomal dominant *diabetes insipidus* to the group of neurodegenerative diseases associated with protein aggregation and provide a biochemical and morphological basis for its pathogenesis.

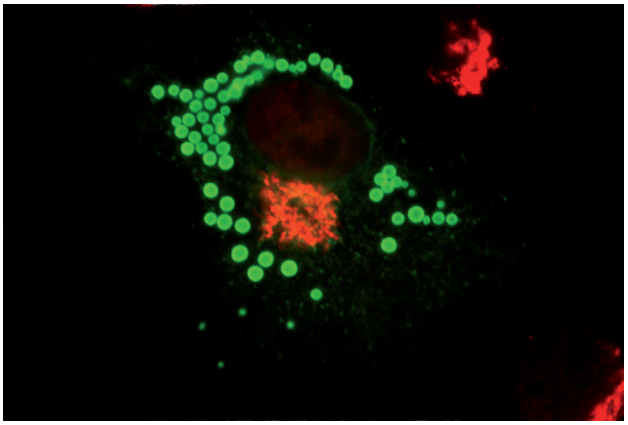

Fig. 2: Granule-like structures generated by wild-type provasopressin (green) in COS cells (stained red for the Golgi marker giantin).

In the course of these experiments, we found that expression of wild-type provasopressin as well as several other regulated secretory proteins in non-endocrine cells is sufficient to generate post-Golgi structures that resemble secretory granules morphologically and biochemically. The minimal mechanism to generate granule-like structures thus does not require an endocrine-specific machinery. Deletion analysis of chromogranin A showed the same segments to be sufficient for granule-like structure formation as are required for granule sorting in endocrine cells, suggesting that self-aggregation in the *trans*-Golgi is at the core of granule formation and sorting into the secretory pathway.

Publications

Meyer, D. M., Crottet, P., Maco, B., Degtyar, E., Cassel, D. & Spiess, M. (2005). Oligomerization and Dissociation of AP-1 Adaptors Are Regulated by Cargo Signals and by ArfGAP1-induced GTP Hydrolysis. *Mol Biol Cell* 16, 4745-4754.

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Junne, T., Schwede, T., Goder, V. & Spiess, M. (2006). The plug domain of yeast Sec61p is important for efficient protein translocation, but is not essential for cell viability. *Mol Biol Cell* 17, 4063-8.

Junne, T., Schwede, T., Goder, V. & Spiess, M. (2007). Mutations in the sec61p channel affecting signal sequence recognition and membrane protein topology. *J Biol Chem* 282, 33201-9.

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Focal Area
Infection Biology

Objective

The objective is the understanding of infectious diseases at the cellular and molecular levels and the focus is on bacterial infections. Six groups (C. Arrieumerlou [since March 2006], D. Bumann [since April 2007], GR Cornelis, C. Dehio, U. Jenal [since 2006] & J. Pieters) decipher the pathogenesis of seven different bacterial pathogens: *Bartonella spp*., *Capnocytophaga canimorsus*, *Mycobacterium* (*M. tuberculosis*, *M. bovis*, BCG and *M. smegmatis*), *Pseudomonas aeruginosa*, *Salmonella spp*, *Shigella flexneri* and *Yersinia enterocolitica*. Besides their own medical importance, these pathogens represent archetypes for a number of different virulence mechanisms (intracellular replication, immune evasion, type III secretion, type IV secretion, biofilm formation...) and provide information which could lead to the design of new treatments or vaccines against a whole range of infectious diseases.

By studying these pathogens, the research field includes extracellular and intracellular pathogens, well-recognized and less-known pathogens. The scope of interest of the different groups covers many different facets of the host-pathogen interaction, including microbial genomics, signal transduction in bacteria and in host cells, nanomachines, cellular trafficking, metabolism and persistence of the pathogen inside the host, inflammation. Thus, the groups develop competence in many different and complementary aspects around the central theme of the biology of bacterial infection. The expertise includes microbial and molecular genetics, cell biology, immunology, biochemistry, fluorescence and electron microscopy, microarrays, siRNA and proteomics.

The impact of this Research goes beyond the specific field of microbial pathogenesis, addressing basic questions in Cell and Molecular Biology like signaling, regulatory principles in nanomachine assembly (length control for instance), DNA transfer between microorganisms and the role of protein kinases in intracellular transport processes in macrophages.

Since 2006, the Focal Area forms the core of the "Center for Systems Bacterial Infections" (C-SBI), a scientific node within SystemsX.ch, the Swiss Initiative in Systems Biology. The approach of a number of questions is thus gradually becoming more global, systems-based. To this end, an RNAi screening platform was established in-house. This platform includes an automated liquid handler which allows automated siRNA transfections and an automated epifluorescence imaging station which can be used

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for high content image-based screens. The platform became operational in 2007 and was already used to screen a library of 132 siRNAs in order to identify new signaling components triggered during Shigella infection.

Extensive collaborations are taking place with our colleagues from the Core Programs “Structural Biology and Biophysics“ as well as with colleagues from the medical Faculty, the Swiss Tropical Institute (Basel) that are also active in Infection Biology and the Paul Scherrer Institute from the ETH (Villigen, CH).

Highlights

In 2007, the Pieters’ group reached a breakthrough on two fronts in their search for the mechanisms of virulence of *Mycobacterium tuberculosis*: First, they showed how the host protein coronin 1 influences intracellular trafficking of *M. tuberculosis* inside macrophages and hence determines the survival of the invading *mycobacteria* (*Cell*, 2007, 130: 37-50). Second, in close collaboration with the Paul Scherrer Institute (Villigen, CH), they managed to crystallize a key virulence factor expressed by *M. tuberculosis*, protein kinase G, in complex with a specific inhibitor, (*PNAS*, 2007, 104:12151-6). These results may open novel avenues for the development of anti-tuberculosis treatments.

The Dehio group has described a novel fundamental role for T4S systems in bacterial pathogenesis, namely to facilitate adaptation to new hosts, eventually leading to a reduction of virulence (*Nature Genetics*., in press). In 2006, C. Dehio was awarded the Swiss „Pfizer Forschungs-Preis 2006, Bereich Infektiologie“ and the German „Hauptpreis 2006 der Stiftung der Deutschen Gesellschaft für Hygiene und Mikrobiologie“.

The injectisome is a self-assembling nanomachine that exports its own constituents before exporting proteins that are injected into animal or plant cells. This implies that this nanomachine recognizes sequentially different categories of substrates. In 2007, the Cornelis group identified a protein of the machine which plays a key role in substrate recognition (*Embo J*, 2007, 26:3015-24).

Education

Since 2004, the members of the Focal Area organize a complete graduate teaching cycle in „Infection Biology“ (Cycle A). A series of lectures on „Major Microbial Diseases and Vaccine Development“ (GR. Cor-

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Focal Area
Infection Biology

nelis, C. Dehio & J. Pieters) and on „Microbial Cell Structures and Drug Targets“ (U. Jenal) took place for the second time during the 2007 fall semester. In 2006, C. Arrieumerlou introduced a new course devoted to “Signaling in inflammation“. After a general introduction on innate immunity, this course covers the molecular basis of pro-inflammatory signaling in response to infections. This includes chemotaxis, signaling, pathogen recognition and NF-KB activation mechanisms. Emphasis is put on fundamental properties of cellular signaling with a systems biology approach of inflammation.

The Focal Area also contributed to the teaching in the Master program in “Infectious Diseases, Vaccinology and Drug Discovery“, jointly organized by the Novartis Institute for Tropical Diseases in Singapore (NITD), The University of Singapore, The Swiss Tropical Institute (Basel) and the Biozentrum. Finally, the Focal Area organized two weekly seminars in Infection Biology.

Host signaling upon *Shigella* infection of epithelial cells

Cells make use of an elaborate control system to integrate various outputs from their environment, compute this information and convert it into physiological cell functions. Recent completion of the genome as well as general data on signal transduction suggesting that cells process information using a limited number of signaling molecules, have raised the question of signaling specificity. How can cells achieve specific cellular functions using common signaling proteins or second messengers?

An increasing amount of experimental evidence has led to a dynamic view of signal transduction, which suggests that specificity could be achieved through the dynamic localization and assembly of signaling protein complexes and networks. In order to get more insight into the complexity of signal transduction, we need to consider all the components of a given signaling network and investigate how the dynamics of each of them contributes to the signaling specificity.

We decided to tackle this question by taking a functional genomic approach in the field of infection biology using *Shigella* infection of epithelial cells as a working model. *Shigella* bacteria are Gram-negative bacteria that invade the colonic and rectal epithelium of primates and humans, causing the acute mucosal inflammation of Shigellosis (1.1 million cases result in death every year). They enter enterocytes by injecting bacterial effectors that take locally control of the host actin machinery to promote bacterial engulfment (See Figure1).

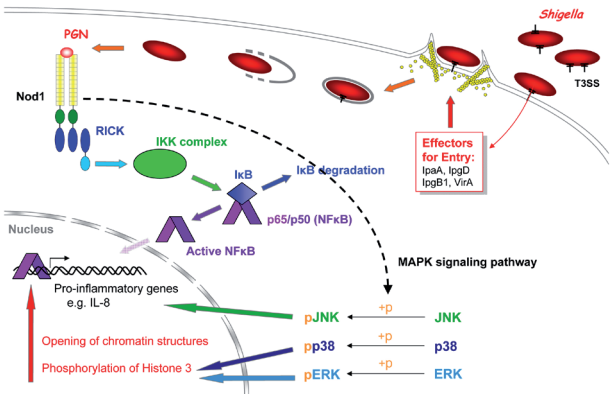

Figure 1: Shigella entry mechanism and host response to the infection (PGN: peptidoglycan).

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In response to bacterial infection, colonic epithelial cells promote a massive inflammatory response via the secretion of pro-inflammatory cytokines such as IL-8. The presence of *Shigella* in the cytosol is detected by the host through Nod1-dependent recognition of bacterial peptidoglycan. This sensing mechanism leads to up-regulation of IL-8 transcription by activating both NF-κB and MAP-kinase pathways. Even though the roles of several host proteins upon infection have been described, we do not have yet an integrated and dynamic model for the signaling network that controls the inflammatory response and its specificity upon infection. Our work has focused so far on the identification of new signaling components involved upon *Shigella* infection and on the propagation of the inflammatory response within the epithelium upon infection.

Characterization of the signaling network induced upon *Shigella* infection (Man Lyang Kim and Hyun Gyeong Jeong)

In order to identify the components of the host signaling network induced upon *Shigella* infection, we decided to use an RNA interference screening approach. Such strategy had been validated in the past by a pilot screen performed at Stanford University using a library of 2400 siRNAs. Our first action was then to set up an equivalent screening platform at the Biozentrum. The platform is equipped with an automated epifluorescence microscope and an automated liquid handler to perform automated transfections and immunofluorescence assays. Based on the results of the pilot screen, 132 were selected and a library of *in vitro* diced siRNAs was generated in our laboratory. Briefly, for each gene of interest, a 600bp region was amplified, *in vitro* transcribed and diced. The siRNA library was then screened in a *Shigella* infection assay optimized for high throughput measurements. To probe the effects of individual gene silencing on *Shigella* uptake and inflammation, the number of bacteria per cell, the number of entry foci and the corresponding level of NF-KB activation were simultaneously measured in populations of single cells using fluorescence microscopy and quantified by automated image analysis. Out of 132 siRNAs tested, we could identify 51 positive and 5 negative hits. Clathrin was identified as the strongest positive hit whereas FGD3 and ARF1 were identified as two strong negative hits.

The roles of these 3 proteins upon *Shigella* infection are currently under investigation in our laboratory. Preliminary results indicated that cells in which Clathrin was silenced showed enhanced level of NF-κB activation in response to *Shigella* infection even though bacterial uptake was similar to control conditions. We could show that NF-κB activation can also be enhanced in response to other NF-KB stimuli such as TNFα and PMA, suggesting that Clathrin can function as a general negative regulator of NF-κB signaling. We are now checking whether this unexpected effect of Clathrin on NF-κB signaling is dependent on its well characterized function in endocytosis. The hypothesis of an endocytosis-independent mechanism would add Clathrin on the list of endocytic proteins for which additional signaling functions have been recently discovered. For FGD3 and ARF1, the reduced NF-κB activation found in the screen upon gene silencing seemed to correlate with a reduction of Shigella uptake. FGD3 contains a Cdc42 specific RhoGEF domain and could therefore interfere on uptake via its action on Cdc42. The protein Arf1, described previously to play a role upon Fc μ receptor-mediated phagocytosis, could also contribute to control membrane trafficking at the site of bacterial entry.

The recent acquisition of a spinning disc confocal microscope will allow us to perform live cell imaging of *Shigella* infections. Using various fluorescent biosensors, we will characterize the signaling dynamics taking place upon bacterial uptake and NF-κB activation.

Propagation of inflammation signaling within the epithelium upon *Shigella* infection (Christoph Kasper and Harry Wischnewski)

This project started from the observation that, upon *Shigella* infection, inflammation signaling propagates from infected cells to uninfected neighboring cells. By performing experiments at low multiplicity of infection, we could observe a strong nuclear translocation of NF-κB in uninfected cells that surrounded infected ones (see Figure2, cells indicated by white arrows). We called this mechanism “transactivation” and made the hypothesis that cell-cell communication could be used by the host organism to alert uninfected tissues of pathogenic invasion. The goal of this project is to characterize the molecular basis of transactivation and to understand its immunological significance upon bacterial invasion.

Transactivation is a general host response to infection. We could observe the same mechanism upon *Listeria* and *Salmonella* infections of epithelial cells. However, in response to these two pathogenic bac-

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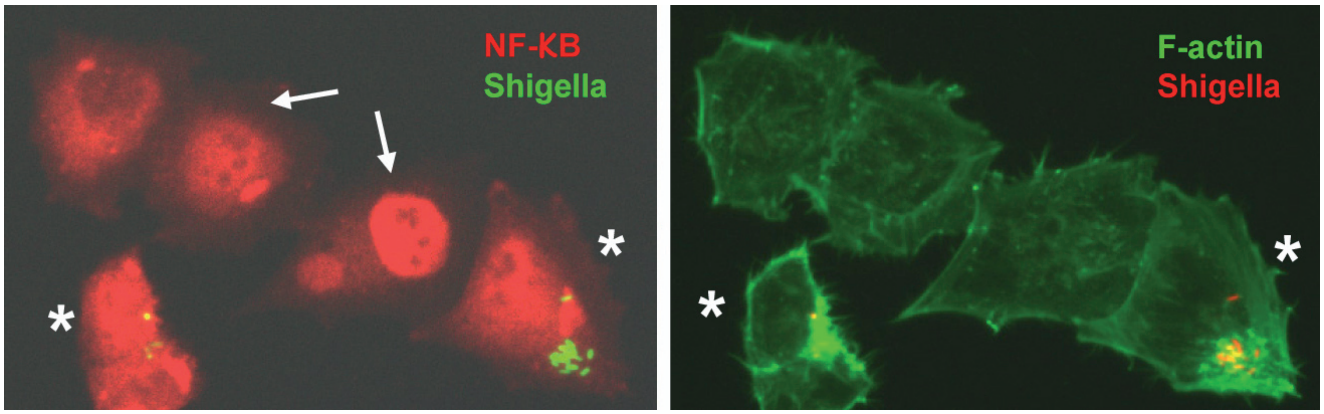

Figure 2: Mechanism of transactivation. Nuclear translocation of NF-κB in uninfected cells (arrows) nearby Shigella infected cells (*). Infected cells (*) showing actin patches at the site of bacterial entry.

teria, efficiency of transactivation was lower and did correlate to the low percentage of infected cells which showed NF-κB activation. Transactivation occurred upon infection of different epithelial cell lines (Hela, Caco-2) as well as in human primary endothelial cells from blood cord. In *Shigella* infected cells, NF-κB is activated in response to the Nod1-dependent detection of bacterial peptidoglycan in the cytosol. This intracellular detection requires that the bacteria invade their host cells and lyse their engulfment vacuole before they can be released in the cytosol and exposed to Nod1. This process takes 15 to 20 minutes. Transactivation occurs shortly (seconds to minutes) after NF-κB activation in infected cells, suggesting that this cell-cell communication mechanism relies on fast signaling events. Transactivation is not dependent on the invasion process itself as the different bacteria tested have different entry mechanisms and since it can be reproduced by microinjecting cells with a Nod1 ligand.

Inter-cellular communication can be mediated by release of a soluble factor, gap junction communication or receptor interactions at the membrane. We could not find any evidence in favor of the first hypothesis. First, transactivation was restricted to cells that were in physical contact with infected cells or other transactivated cells. Second, the supernatant of infected cells did not activate any signaling pathways in control uninfected cells. Third, all molecules tested such as ATP, reactive oxygen species, nitric oxide did not affect transactivation. If these results do not favor the hypothesis of a soluble factor, they do not allow us to totally rule out the possibility that transactivation is mediated by a very locally secreted molecule. In parallel, we tested the possibility that transactivation was mediated by a gap junction communication mechanism. Preliminary results using gap junction inhibitors favor this hypothesis. Treatment of endothelial cells with Carbenoxolone and Heptanol did decrease significantly the level of transactivation upon Shigella infection. Ongoing work is currently done to confirm the hypothesis of a gap junction mediated mechanism and to identify which small molecule might be involved.

To test the immunological significance of transactivation, we tested whether in addition to NF-κB, other pro-inflammatory signaling pathways were also activated in uninfected cells neighboring infected ones. We could observe that this was the case for JNK and P38 MAP kinase pathways. Similar to what we observed for NF-κB activation, the nuclear phosphorylation of P38 was even higher in transactivated cells than in infected cells. The low activation of NF-κB and p38 in infected cells can probably be explained by the activities of two bacterial effectors OspG and OspF. Indeed, the group of P. Sansonetti has shown that Shigella has developed strategies to interfere with inflammation signaling in infected cells. For instance, the bacterial effector OspG can alter NF-κB activation by interfering with the degradation of IκB, the inhibitor of NF-κB. In addition, the effector OspF prevents the phosphorylation of P38 in the nucleus. What we showed for the first time was that the host has probably developed a cell-cell communication strategy to circumvent the effects of bacterial effectors that only function in infected cells. By propagating inflammation signaling to neighboring cells, transactivation would ensure that inflammation can still be induced upon infection. We directly tested this hypothesis by visualizing at the single cell level the amount of IL-8 produced. Very interestingly, we could show that transactivated cells produce much more IL-8 than infected cells.

Together, these results suggest that transactivation is an efficient host defense mechanism to circumvent the bacterial effector activities and to ensure inflammation signaling and IL-8 production upon bacterial infection. This inflammation amplification phenomenon might explain, at least to a certain extent, the massive inflammation observed in the colonic epithelium of patients infected by Shigella.

Publications

Kasper C., Wischnewski H. & Arrieumerlou C. Propagation of inflammation signaling as a host defense mechanism against bacterial infection of the epithelium. *Manuscript in preparation*.

Thedieck K., Polak P., Kim M.L., Molle K.D., Cohen A., Jenö P., Arrieumerlou C. & Hall M.N. (2007) PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis, *PloS ONE*, *Online publication* Nov. 21st.

Oleg D., Hagren O.I., Sagetorp J., Arrieumerlou C., Gylfe E., Tengholm A. (2007) Glucose metabolism generates cyclic AMP oscillations that regulate pulsatile insulin secretion. *Cell metabolism, In revision*.

Salmonella-host interactions

Infectious diseases represent a major worldwide threat to human health. Novel strategies to combat infectious disease are urgently needed because of rising resistance of pathogens to antimicrobial therapy, an increasing number of immunosuppressed patients that are highly susceptible to infection, increasing travel which enhances transmission and worldwide spread of novel and re-emerging pathogens, and potential bioterrorism threats.

The substantial progress in infection biology research in the last two decades could provide a basis for novel control strategies. In particular, genomes of all major human pathogens as well as human and animal model hosts have been sequenced, a large number of pathogen virulence factors as well as antimicrobial immune mechanisms have been identified and understood in molecular to atomic detail, and pathogen as well as host responses have been studied using global approaches such as transcriptomics and proteomics. However, it has remained difficult to translate this extensive knowledge into effective new control strategies. Specifically, efficacious vaccines are still lacking for major pathogens, and the dramatic decline in development of novel antimicrobials over the last 20 years is likely to substantially reduce treatment options in the near future.

One potential reason why it is so difficult to translate basic research to effective strategies for combating infectious diseases, could be the prevailing focus on the action of individual pathogen or host components. While this reductionist approach was highly successful to identify and characterize key virulence and immune factors, it can not explain the course of complex multifactorial infectious diseases involving hundreds of interacting pathogen and host factors. Integration of the vast existing knowledge and development of appropriate methodology to analyze interacting host/pathogen networks will be required to facilitate rational development of new control strategies.

Such a system-level approach is technically challenging since hundreds of pathogen and host factors need to be simultaneously analyzed. Moreover, *in vivo* infection models are more appropriate than *in vitro* cell culture infection models to mimic important aspects of dynamic inflammation processes of human disease, but the high *in vivo* complexity imposes additional challenges. An integrated approach to infection is also conceptually difficult because two systems (pathogen and host) and their interactions need to be understood. The new field Systems Biology is providing novel concepts and methodology

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that could help to meet these challenges. In fact, infection is a particularly fascinating research area for Systems Biology since one can study interactions of two co-evolved systems with major importance for human health.

The major human and veterinary pathogen *Salmonella* is an excellent model pathogen for this type of research because of extensive available genome data, close relatedness to *E. coli* enabling the use of sophisticated tools generated for this prime model organism, simple cultivation and molecular biology, availability of mouse disease models that mimic important aspects of human typhoid fever or enteritis, and large knowledge bases including reported mouse virulence phenotypes for some 200 different *Salmonella* mutants.

FACS purification of *Salmonella* from infected host tissues

System-level *in vivo* analyses require more than thousand fold enrichment of *Salmonella* from the excess of host material in infected tissues. We achieved this by FACS-sorting *Salmonella* expressing the green fluorescent protein (GFP) from infected tissues (Becker, Selbach *et al.* 2006; Bumann and Valdivia 2007; Thöne, Schwanhäuser *et al*. 2007) (Fig. 1). Critical for successful purification is the suppression of interfering host autofluorescence enabling sensitive detection of *Salmonella* expressing low non-attenuating GFP levels, and the availability of high-speed sorters equipped for aerosol management appropriate for BSL 2 infectious samples.

These technical developments enabled us to identify more than 150 different *Salmonella* promoters that are highly active in infected mouse spleen or during enteritis from a random promoter-trap library using a differential fluorescence induction approach. We have characterized these promoters in two different disease models (enteritis and typhoid fever) as well as *in vitro* (Rollenhagen and Bumann 2006). These data provided detailed new insights into major *Salmonella* activities during infection. One particular surprising finding was the disease-specific activity of most strong promoters.

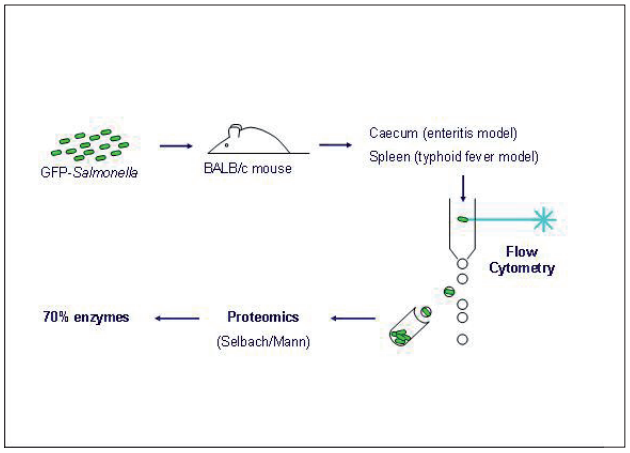

Fig. 1. Purification and analysis of Salmonella from infected mouse tissues.

Further improvements in flow cytometry enabled us to purify *Salmonella* from infected mouse tissues in sufficient amounts and purity for proteome analysis in collaboration with Matthias Selbach and Matthias Mann. This first *ex vivo* proteome analysis of any pathogen provided a system-level overview of *Salmonella in vivo* activities with unprecedented detail (Becker, Selbach *et al*. 2006). Interestingly, some 70% of the identified highly abundant proteins were metabolic enzymes suggesting that metabolism was a major activity of *Salmonella* during infection. Metabolism has previously not been considered to be of major relevance to infectious diseases since large-scale genetic screens in various pathogens found only very few enzymes to be required for virulence.

System-level analysis of *Salmonella* metabolism during infection

The apparent discrepancy of *in vivo* prevalence of metabolic enzymes but weak phenotypes of many individual metabolic mutations, might be partially explained by the well-known robustness of metabolic networks that are largely resilient against single enzyme defects. To investigate this hypothesis in more detail, we performed a system-level analysis of *Salmonella in vivo* metabolism (Becker, Selbach *et al*. 2006) (Fig. 2). Specifically, we integrated *in vivo* proteome data, genome comparisons, literature data, and *in vivo* phenotypes of 30 novel mutants into a comprehensive graphical model of *Salmonella* metabolism in infected mouse spleen. This model demonstrated a remarkable metabolic robustness since only 126 of 534 functionally characterized enzymes were essential for *Salmonella* virulence. Two factors largely explained this robustness: (i) internal redundancy of *Salmonella* metabolism and (ii) availability of a large variety of host nutrients. Our findings had major implications for selection of targets for development of novel antimicrobials. On this basis, we have initiated a large-scale small molecule screening in a project funded as part of the Go-Bio award of the German Ministry of Education and Research.

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Vaccine development

Fig. 2: Overview of Salmonella metabolism during typhoid fever. Symbols represent metabolites and connecting lines represent enzymes catalyzing the corresponding reactions. Detected in vivo enzyme expression (yellow) and in vivo enzyme relevance (red, essential; orange, contributing to virulence; blue, dispensable), indirectly inferred enzymes and metabolites (white), and metabolites and enzymes with no available in vivo evidence (grey) are shown. Brown lines represent the two Salmonella membranes.

Besides antimicrobial chemotherapy, vaccination can offer effective control of infectious diseases. Subunit vaccines containing only a few immunogenic components of a pathogen are increasingly substituting classical whole-cell vaccines because of less adverse side effects and better quality control. However, for complex bacterial pathogens and parasites, the identification of promising antigenic components is a challenging task since usually only a few percent of several thousand antigen candidates are suitable. Using our *in vivo* technologies we had previously shown that CD4 T cells crucial for immunity to intracellular pathogens preferentially recognize *Salmonella* antigens that are highly expressed during infection. Based on these findings, we could identify the first two highly protective *Salmonella* antigens among an early set of five highly expressed *Salmonella* proteins. To identify additional promising antigen candidates, we developed together with Dennis Kirchhoff and Alexander Scheffold, DRFZ Berlin, a sensitive method to detect CD4 T cells against *Salmonella* antigens in *Salmonella*-infected mice (Kirchhoff, Frentsch *et al*. 2007). We combine this technique with our recent *Salmonella in vivo* proteome data set to identify well recognized antigens that could represent protective antigens. A combination of the best antigens will enable us to develop an effective typhoid and paratyphoid fever vaccine to control these major infectious diseases that kill several hundred thousand children each year. Currently available vaccines induce only moderate levels of protection against typhoid fever and are ineffective against paratyphoid fever.

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The *Yersinia* injectisome and pathogenesis of *Capnocytophaga canimorsus*

Type III secretion injectisome

We are studying type III secretion (T3S), a complex weapon, encountered in more than twenty animal, plant and insect Gram-negative pathogens. It consists of a nanosyringe related to the flagellum (the injectisome) and an array of effector proteins. Bacteria docked at the surface of a eukaryotic host cell or included within a vacuole inject the effectors across the cellular membrane, into the cytosol of the host cell (Cornelis and Wolf-Watz, *Mol Microbiol*. 1997). The archetypal T3S system of *Yersinia* (*Y. enterocolitica* and *Y. pestis*), consists of the Ysc injectisome and the Yop effectors. The Yop effectors are injected into macrophages and dendritic cells where they interrupt the signalling cascades leading to phagocytosis and to the onset of the inflammatory response. The injectisome serves as a hollow conduit through which the Yop effectors travel, unfolded and in one step across the two bacterial membranes and the peptidoglycan. It is made of 27 Ysc proteins and consists of a cylindrical basal body anchored to the inner and outer membranes joined by a central rod and supporting a hollow needle about 7 nm thick and 67nm long. The needle, which terminates with a tip structure made of protein LcrV (Mueller *et al*., *Science*, 2005), does not puncture the target cell membrane but, upon contact, inserts a „translocation pore“ made of the two hydrophobic proteins YopB and YopD. The basal body contains five transmembrane proteins which form the core of the export machine. After its assembly, this machine exports itself the extracellular portion of the injectisome. The same machine thus exports sequentially the needle constituants, the pore-formers and the Yop effectors meaning that it changes its substrate-specificity over time. The length of the needle is genetically controled and correlates with the size of the protein YscP (Journet *et al*., *Science*, 2003; Mota *et al*., *Science* 2005). Our current model is that YscP acts both as a ruler and a substrate specificity switch (T3S4 domain). When this protein „senses“ that the needle has reached its final length, its C-terminus transmits a signal to the export machine, which then changes its substrate specificity and stops exporting needle subunits (Fig. 1).

During the 2004-2005 period, our work focused mainly on the structure and assembly of the needle and of the translocation pore. By quantitative immunoblot analyses and scanning transmission electron microscopy (STEM), we determined that the needle of the *Y. enterocolitica* E40 injectisome consists of 139 +/- 19 YscF subunits and that the tip complex is formed by three to five LcrV monomers. A pentamer

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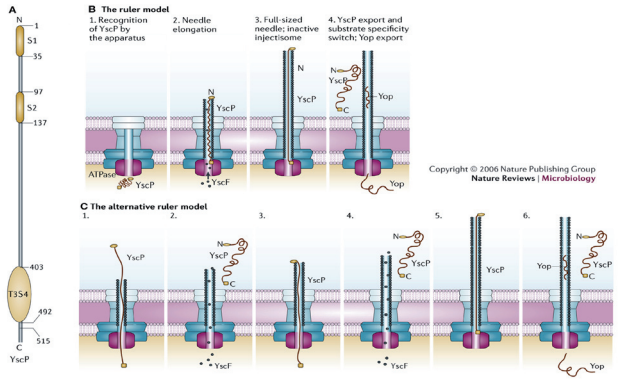
Fig 1 : The ruler model for control of the injectisome needle length.



A. Schematic representation of YscP, showing the export signals localized in the N-term part and the T3S4 domain localized in the C-term part of the protein.
B. YscP enters the channel after completion of the basal structure 1. The needle subunits are exported and polymerize, leading to the extension of the ruler part of YscP 2. The needle assembly is completed 3. The T3S4 domain of YscP switches the substrate specificity 4. and the ruler is released.
C. An alternative ruler model. In this, more dynamic model, ruler and subunit molecules are exported alternatively (1. to 4.) The ruler checks the length while travelling through the channel (1. and 3.). When the exact length is reached, the ruler switches the substrate specificity 5. and leaves, leaving the injectisome ready for Yops export 6. Taken from Cornelis, GR Nature Reviews Microbiology 4: 811-825, 2006.

represented the best fit for an atomic model of this complex (Fig. 2C.) Hybrid proteins were made between LcrV and its orthologs PcrV (*P. aeruginosa*) or AcrV (*A. salmonicida*). All hybrid proteins could form tip structures on the *Y. enterocolitica* needles and the analysis of these tips by STEM allowed to determine the orientation of the known LcrV 3-D structure (Derewenda *et al*., *Structure*, 2004) in the tip complex: the N-terminal globular domain of LcrV forms the base, while the central globular domain forms the head (Fig 2AB). Recombinant *Y. enterocolitica* expressing some hybrid tips could form the translocation pore while others could not. There was a good correlation between formation of the translocation pore, insertion of YopB into target cell membranes and interaction between YopB and the N-terminal globular domain of the tip complex subunit. Hence, the base of the tip complex appears to be critical for the insertion of a functional YopBD pore into the host cell membrane.

This pore is required for the injection of the effectors. However, we observed that its insertion into target cells also triggers the release of pro-inflammatory signals. This counter-productive effect is neutralized by the injection of anti-inflammatory effectors. Our observation was the following: while macrophages infected by wild type *Y. enterocolitica* did not release mature IL-1β, macrophages infected by *Y. enterocolitica* deprived of all effectors released mature IL-1β. Surprisingly, macrophages infected by *Y. enterocolitica* deficient for secretion of all T3S proteins, including effectors and translocators, did not release mature IL-1β. Using different genetic constructs, we showed that insertion of T3S translocation pore triggers activation of caspase-1, maturation of proIL-1β and release of mature IL-1β, which occurs independently of cell osmotic lysis. These data thus show that T3S translocation is intrinsically a pro-inflammatory phenomenon.

YscU is one of the five integral transmembrane proteins forming the export apparatus of the injectisome. It consists of four N-terminal alpha helices and a long cytoplasmic C-terminal domain, which undergoes auto-cleavage at a NPTH site. We prevented autocleavage by substitutions N263A and P264A. As a result, export of LcrV, YopB and YopD was abolished but export of the Yop effectors was unaffected. As a consequence, *yscU*N263A mutant bacteria made needles without the LcrV tip complex and they could not form translocation pores. The graft of the export signal of the effector YopE, at the N-terminus of LcrV, restored LcrV export and assembly of the tip complex. Thus YscU cleavage is required to acquire the conformation allowing recognition of translocators, which represent an individual category of substrates in the hierarchy of export. In addition, *yscU*N263A mutant bacteria exported reduced amounts of the YscP ruler and made longer needles. Increasing YscP export resulted in needles with normal size, depending on the length of the ruler. Hence, the effect of the *yscU*N263A mutation on needle length was the consequence of a reduced YscP export.

Capnocytophaga canimorsus

In 2004, we initiated the study of the pathogenesis of *Capnocytophaga canimorsus*, a Gram-negative bacterium from the Order of *Flavobacteria*, commonly found in dog‘s mouths. It is responsible for fatal septicemia or meningitis after dog bites. An overview of case reports suggests that the bacterium induces little inflammation during the early stages of the infection, until overwhelming bacterial growth and spread finally causes shock or meningitis. We characterized

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the proinflammatory response of macrophages and epithelial cells to infection by *C. canimorsus*. We found that, indeed, *C. canimorsus* bacteria are not recognized by toll-like receptor 4 and hence do not trigger an inflammatory response. Moreover, one strain even actively blocked the pro-inflammatory response induced by other bacteria. To go further in our investigation, we developped genetic tools (conjugation, transformation, expression vectors)

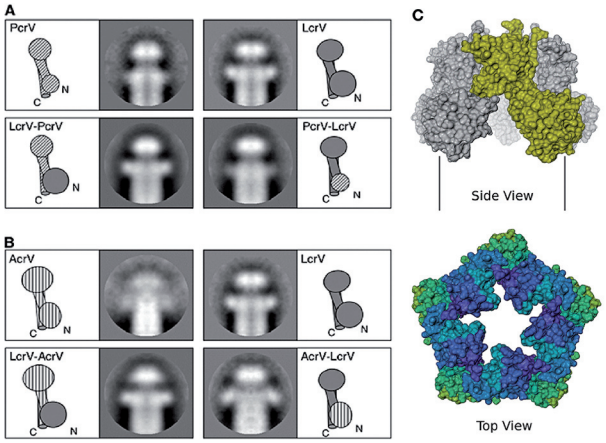

Fig. 2: Tip complexes formed by Y. enterocolitica ΔHOPEMNV complemented with the four hybrid proteins mentioned. Samples stained with 2% (w/v) sodium phosphotungstate were imaged by dark-field STEM at a resolution of 1.5 nm. A. Projection averages of tip complexes formed by wild-type PcrV and LcrV,(top) and the LcrV–PcrV, PcrV–LcrV hybrids (bottom). Schematic representations of all proteins are drawn next to the corresponding tip complexes. B. Projection averages of tip complexes formed by wild-type AcrV and LcrV, (top) and the LcrV–AcrV, AcrV–LcrV hybrids (bottom). Schematic representations of all proteins are drawn next to the corresponding tip complexes. Number in brackets indicate the number of projections taken to calculate the respective average. Width of frames is 20 nm. C. Model of the tip structure. Side view and top view of the pentameric LcrV tip complex model at the top of the injectisome needle (represented by two vertical lines, separated by 8.2 nm). Modelling was performed using the visualization tool DINO (http://www.dino3d.org); the molecular surfaces were calculated with MSMS. In the side view, a single monomer is highlighted in yellow. In the top view, a colour gradient has been applied along the chain for aesthetic reasons. Details available upon request. Taken from Broz et al., Molecular Microbiology 65: 1311-1320, 2007.

and generated a transposon mutants library. We also pursued our effort to sequence the genome of *C. canimorsus* 5 (Cc5). Data from sequencing a 4-kb inserts plasmid library (3.5 read coverage), a 40-kb fosmid library (0.6 read coverage) and pyrosequencing (20 read coverage), completed by primer walking allowed to assemble the genome in two contigs and one circular scaffold. The genome has a size of 2.573 Mb, reminiscent of the size of several commensal or pathogenic bacteria from the upper respiratory tract. The annotation of 2474 putative coding sequences is in progress.

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Functional analysis of type IV secretion systems in bacterial pathogenesis

The aim of our studies is to gain a molecular understanding of the function of type IV secretion (T4S) systems in bacterial pathogenesis. T4S systems are ancestrally related to bacterial conjugation systems, which mediate the efficient spread of genetic traits such as antibiotic resistance genes among bacterial populations. Bacterial pathogens targeting eukaryotic host cells have adopted T4S for the intracellular delivery of virulence factors, such as effector proteins or DNA, that modify host cellular functions in favor of the pathogen. A supramolecular assembly of 11 proteins is thought to span both Gram-negative bacterial membranes and the host cell membrane, allowing translocation of the virulence factors from the bacterial cytoplasm directly into the host cell cytoplasm. Our previous work on the pathogenesis of the vascular tumor-inducing bacteria of the genus *Bartonella* revealed central roles of two distinct T4S systems, VirB/VirD4 and Trw, in the ability of bacteria to colonize, invade and persist within vascular endothelial cells and erythrocytes, respectively (reviewed in Dehio, 2005, *Nat. Rev. Microbiol*. 3, 621-631). We have shown that the VirB/VirD4 T4S system of *Bartonella henselae* mediates the subversion of multiple endothelial cell functions related to vascular tumor formation, including (i) inhibition of apoptotic cell death, resulting in enhanced endothelial cell survival, (ii) massive rearrangements of the actin cytoskeleton, resulting in the formation and internalization of large bacterial aggregates by a unique structure known as the invasome, and (iii) nuclear factor kappa B-dependent pro-inflammatory activation, leading to cell adhesion molecule expression and chemokine secretion. Further, we have shown that all these effects are mediated by the VirB/VirD4-dependent injection of a cocktail of seven bacterial effector proteins termed BepA-BepG (Bep = *Bartonella*-translocated effector protein). These effectors carry a bipartite secretion signal at their C-terminus, which is composed of the BID (Bep intracellular delivery) domain and a short positively-charged tail sequence. The N-terminal parts of the Bep effectors carry diverse domains or peptide motifs that are considered to mediate effector functions within host cells (Fig. 1). For instance, upon translocation the effector proteins BepD, BepE and BepF become tyrosine phosphorylated in short N-terminal repeat motifs and thereby potentially interfere with eukaryotic signal transduction processes (Selbach *et al.*, submitted). Defining the cellular targets and molecular mechanisms of how individual effectors proteins interfere with eukaryotic signaling processes have become a focus of our recent studies. At present, we are most advanced with the functional analysis of BepA and BepG.

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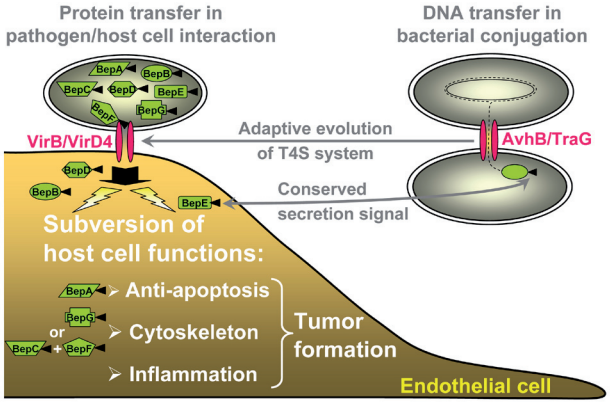

Figure 1: Effector proteins translocated by the Bartonella T4S system VirB/VirD4 subvert human endothelial cell functions.

BepA inhibits apoptosis by elevating intracellular levels of cyclic-AMP

The capacity of *B. henselae* to inhibit the apoptosis of human vascular endothelial cells was found to be entirely dependent on the effector protein BepA. Upon translocation, BepA localizes to the plasma membrane, where it triggers the production of the second messenger cyclic AMP (cAMP) by a yet unknown mechanism. The resulting increase in the steady-state concentration of cAMP blocks effectively apoptotic cell death. The BID domain of BepA is sufficient to mediate membrane localization, elevation of cAMP levels and the resulting protection from apoptosis. This approximately 140 aa long domain, which evolved in bacteria as a part of a composite T4S signal, has thus acquired an additional effector function within host cells (Schmid *et al*., 2006).

BepG triggeres F-actin rearrangements that result in a specific mode of bacterial uptake

The effector protein BepG was found to inhibit endocytic uptake of individual *B. henselae* bacteria by human vascular endothelial cells, thereby redirecting bacterial uptake to the so-called invasome-mediated pathway resulting in the internalization of large bacterial aggregates. Invasome-mediated uptake is facilitated by massive rearrangements of the F-actin cytoskeleton that depend on the small GTPase Rac1, the Rac1-effector Scar1, and the F-actin organizing complex Arp2/3 (Rhomberg *et al.*, submitted). While BepG is the only T4S effector sufficient to trigger invasome-mediated uptake, the synergistic action of BepC and BepF have a similar outcome. As BepC, BepF and BepG have diverse domain structures we assume that the redundancy in triggering this unique mode of bacterial entry is the result of targeting different, yet converging signal transduction pathways.

Genomic analysis of *Bartonella* identifies T4S systems as host adaptability factors

A different line of research allowed us to identify a novel and unexpected role for T4S systems in the evolution of the pathogenic genus *Bartonella*. The 21 species of that genus have evolved in two lineages that display marked differences in the adaption to their specific host(s) and the opposing degree of virulence. The “ancestral” lineage is comprised of a single species, *Bartonella bacilliformis*, that causes life-threatening infections in humans and thus is only poorly adapted to its host. All other species evolved as benign, host-adapted pathogens in a modern lineage by radiating speciation. We have used comparative and functional genomics to infer pathogenicity genes specific to the modern lineage, and we suggest that these genes may have facilitated adaptation to the host environment and a concomitant reduction in virulence. We determined the complete genome sequence of *Bartonella tribocorum* and functionally identified by transposon mutagenesis 97 pathogenicity genes that are required for infectivity of this modern species in an animal model. Eight-one of these 97 pathogenicity genes are shared by the species of the modern lineage. Sixty-six of these 81 pathogenicity genes are present in *B. bacilliformis* and one has been lost by deletion in this representative of the ancestral lineage. The 14 pathogenicity genes that are specific for the modern lineage encode components of either the VirB/VirD4 or Trw systems, suggesting that these horizontally acquired T4S systems have a prominent role in host adaptability (Saenz *et al*., 2007). We assume that the role of T4S systems in host adaptability inferred here for the genus *Bartonella* might be a shared feature of effector-translocating T4S systems in other host-associated bacteria.

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Role of cyclic di-GMP signaling in bacterial persistence

Although many microbial pathogens causing acute traumatic diseases have been successfully countered with vaccines and antibiotics, they have been replaced by environmental microorganisms, which have gained a foothold in the human body. These new microbial enemies often colonize compromised organs in protected biofilm structures where they resist antibiotic treatment and host defense mechanisms. Internal reservoirs of persistent microorganisms cause inflammation and chronic diseases, and can serve as base for recurrent acute attacks on the host as exemplified by urinary tract infections, cystic fibrosis or infections associated with prosthetic implants. We have initiated studies that aim at understanding the role of the second messenger cyclic di-GMP in chronic infections and bacterial persistence. The recent finding that c-di-GMP signaling plays a critical and possibly widespread role in bacterial virulence and persistence underscores the necessity to carefully analyze and define c-di-GMP signaling principles in pathogenic bacteria.

To find potential environmental or intracellular cues that control biofilm formation we used an *Escherichia coli in vitro* model system, which reflects important aspects of urinary tract infections, to screen a chemical compound library for substances that alter biofilm formation. Substances that inhibit bacterial translation – including clinically relevant antibiotics – lead to a strong induction of biofilm formation. Analysis of the molecular mechanisms underlying this remarkable phenomenon identified the components of a signal transduction cascade that senses the translational performance of the ribosome and links it to biofilm formation. This signal transduction cascade was shown to rely on specific c-di-GMP signaling proteins and on another bacterial second messenger, guanosine-bis 3´, 5´(diphosphate) (ppGpp). Together ppGpp and c-di-GMP orchestrate the synthesis of a specific polysaccharide adhesin that is an essential component of the biofilm matrix in our model system. These studies identify several new cellular components that are essential for biofilm induction. This knowledge will be exploited to screen for drugs that specifically target bacterial persistence and biofilm formation.

In parallel we have initiated studies that aim at understanding the role of the second messenger c-di-GMP in chronic infections of the opportunistic human pathogen *Pseudomonas aeruginosa*. Our work has focused on the relationship between c-di-GMP regulation and the *P. aeruginosa* small colony variant (SCV) phenotype. SCVs are highly adherent, auto-aggregative morphotypes associated with per-

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sistence, poor lung function, and enhanced antibiotic resistance in cystic fibrosis lung infections. A comprehensive transposon screen for SCV-associated genes identified several components of the c-di-GMP network, supporting the hypothesis that c-di-GMP regulation is critically associated with SCV. We are currently analyzing several of these signaling systems in detail to determine their role in *P. aeruginosa* lung persistence, as well as their input signals, activation mechanisms, and downstream targets. These studies will lead to a better molecular understanding of a model case for bacterial persistence.

Analyzing the Interaction of Pathogens with the Host Immune System

Introduction

Activation of the immune system against pathogenic organisms involves the internalization of infectious material into macrophages, the processing and presentation of pathogen-derived peptides to T lymphocytes and the activation of these T lymphocytes to carry out effector functions. Both the internalization and processing of pathogens, as well as activation of T cells through recognition of infected macrophages is regulated through signal transduction processes.

Many pathogenic microorganisms have gained the capacity to circumvent the effectiveness of the immune response at several levels, and a major focus of the laboratory is to decipher the mechanisms that are used by pathogens to escape immune recognition. In particular, we are studying the survival mechanisms of the important bacterial pathogen *Mycobacterium tuberculosis*.

Interaction of Pathogenic Mycobacteria with Mammalian Cells

Several microorganisms can invade and survive within mammalian host cells. Mycobacteria have the ability to enter and survive within eukaryotic cells, by preventing phagosome-lysosome fusion, making this an excellent model system to study both the pathogen-host interaction as well as the biochemical events involved in regulating transport events inside the host cell. We are interested in both the host as well as the mycobacterial factors contributing to mycobacterial survival.

Mycobacterial Factors involved in the Modulation of Intracellular Trafficking

We are analyzing the bacterial factors that allow pathogenic mycobacteria to survive within macrophages. We found that mycobacteria utilize a eukaryotic-like serine/threonine kinase, termed protein kinase G (PknG) to subvert lysosomal delivery and ensure their intracellular survival (see Walburger *et al.*, Science (2004) vol. 304:1800). Blocking PknG either chemically or through gene deletion in *M. tuberculosis* results in the rapid lysosomal delivery and killing of *M. tuberculosis*. Thus, pathogenic mycobacteria have evolved eukaryotic-like signal transduction mechanisms capable to modulate host cell trafficking pathways. In order to further understand the mode of action of protein kinase G, PknG was crystallized in the presence of a specific inhibitor and the structure was determined at 2.4 A resolution (see Figure 1).

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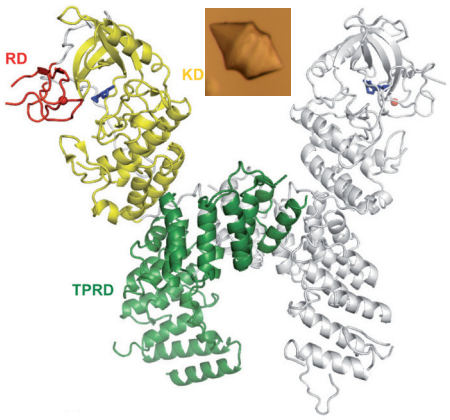

Figure 1. Structure of the PknG dimer from M. tuberculosis at 2.4 A resolution. The crystal structure was obtained through co-crystallization of PknG with a specific inhibitor. Inset: A crystal of PknG from Mycobacterium tuberculosis. From: Scherr et al., PNAS (2007), vol. 104, 12151-6

The structure revealed that PknG contained a unique N-terminal domain that is not found in any human kinase, and explained the specificity of chemical inhibition of protein kinase G (Scherr et al., 2007). Ongoing work aims to determine the signal transduction pathway in which protein kinase G operates in order to ensure the establishment of an intracellular infection of *M. tuberculosis*.

Macrophage Factors involved in the Modulation of Intracellular Trafficking

Analysis of the constituents of mycobacterial phagosomes revealed the accumulation of a host protein, termed coronin 1 or TACO (for *t*ryptophan *a*spartate containing *c*oat *p*rotein) that is a crucial factor for mycobacterial survival inside macrophages. In the absence of coronin 1, mycobacteria are readily transferred to lysosomes (Ferrari *et al.*, *Cell* (1999) vol. 97:435 and Gatfield and Pieters, *Science* (2000) vol. 288:1647).

To understand the normal function of coronin 1 *in vivo*, we have generated macrophages as well as mice devoid of coronin 1. When coronin 1-deficient macrophages are infected with mycobacteria, the bacilli are rapidly transported to lysosomes and killed, demonstrating an essential role for coronin 1 in intracellular mycobacterial survival Jayachandran *et al*. 2007a, 2007b, see Figure 2).

Apart from the inability of mycobacteria to survive in coronin 1 deficient macrophages, mice lacking coronin 1 develop and reproduce normally, are viable

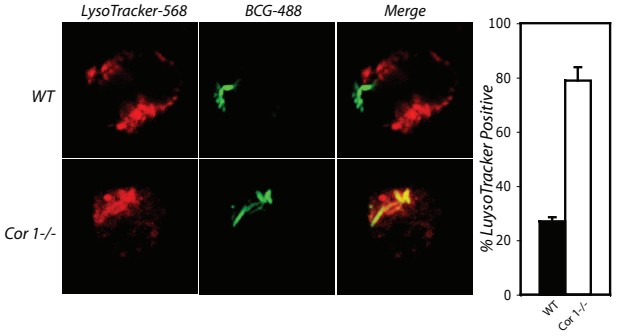

Figure 2: Intracellular trafficking and survival of mycobacteria. Macrophages from wild type or coronin 1 deficient mice were infected with M. bovis BCG after which the intracellular localization was assessed (anti-BCG antiserum: green; lysosomal marker lysotracker: red). From: Jayachandran et al., Cell (2007), vol. 130, 37-50.

and show no obvious defect. Additionally, coronin 1 deficient macrophages are fully functional and show no defect in phagocytosis, macropinocytosis nor cell motility.

We found that while mice lacking coronin 1 are perfectly healthy, coronin 1 is required for the activation of the Ca^{2+} dependent phosphatase calcineurin in macrophages (Jayachandran *et al.*, 2007a). In normal macrophages, upon internalization of mycobacteria, this phosphatase becomes activated, thereby blocking phagosome-lysosome fusion by an as yet unknown mechanisms and allowing mycobacterial survival. In the absence of coronin 1, calcineurin activation does not occur, resulting in phagosome-lysosome fusion and intracellular killing of the internalized mycobacteria. Strikingly, the genetic depletion of coronin 1 can be phenocopied by the addition of the calcineurin inhibitors cyclosporin A and FK506. Thus, it appears that coronin 1 has evolved to activate Ca^{2+} dependent signaling reactions in macrophages thereby promoting the survival of pathogenic mycobacteria.

Our current work focuses on understanding the molecular mechanisms of coronin 1-mediated signaling.

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Focal Area Neurobiology

Mechanisms controlling the development and function of the central nervous system

A major challenge in biology is to understand how the nervous system is assembled to appropriately respond to a large range of stimuli and control complex functions such as behavior and emotions. The nervous system must be capable of storing, integrating and retrieving information using mechanisms that are still poorly understood, but surely dependent on the establishment of precise connections. How neurons are generated to then form meaningful functional circuits is an intriguing question that is not only of interest to developmental neurobiologists, but also of great significance in the context of diseases, injury of the nervous system or perturbations of body weight regulation. There is little growth of axons in the central nervous system (brain and spinal cord) after injury in the adult, and when neurons die or fail to efficiently contact other cells, the brain is unable to store or retrieve memories, as illustrated for example in the disease of Alzheimer, a condition that affects a large proportion of the aging population in industrialized countries. The fine-tuning of synaptic connections and the homeostasis between excitatory and inhibitory inputs is also frequently perturbed in neurological diseases, including epilepsy in particular. A better knowledge of the mechanisms controlling the assembly and function of neuronal networks is thus desirable as it will significantly contribute to the understanding of diseases of the nervous system.

Education

The members of the Focal Area organize and teach a graduate and post-graduate program in Neurobiology. This includes a series of formal introductory lectures in Neurobiology, a 3-week intense program including practical work, as well as a Journal Club series both at the FMI and at the Biozentrum. In addition, a weekly, 2-h lecture series is organized on Current Topics in Neurobiology and weekly seminars with invited speakers. This teaching program includes the active participation of a large number of members of the Basel Neuroscience Program, in particular neurobiologists working at the FMI and for pharmaceutical companies. More information on the program at www.biozentrum.unibas.ch/neuro

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Neuronal circuit assembly and function in the developing spinal cord

The aim of our studies is to understand the molecular and mechanistic basis involved in the establishment of specific neuronal connections within a circuit of interconnected neurons. A deep knowledge about the logic of how neuronal circuits assemble during development and which molecules control the establishment of neuronal circuits will contribute to our understanding of maturation, functioning and plasticity of the nervous system.

Our projects focus on the molecular and cellular mechanisms controlling the specification of neuronal circuits implicated in motor control. Studies on circuits controlling motor behavior offer the unique property of revealing a direct link between fine details of dedicated circuit architecture and an immediate behavioral output. In particular, we are interested in understanding how selectivity of connections in neuronal circuits of the spinal cord allows temporally and spatially precise activation of motor neurons leading to appropriate motor behavior, the ultimate behavioral output of all nervous system activity.

In the spinal monosynaptic reflex circuit (Figure 1), motor neurons in the ventral horn of the spinal cord project to innervate extrafusal muscle fibers in the periphery. Motor neurons receive sensory feedback through group Ia proprioceptive afferents innervating muscle spindles peripherally and establishing projections into the ventral spinal cord centrally (Figure 1). The elucidation of mechanisms controlling the establishment of specific sensory-motor connections has represented a major goal of our recent studies. To unravel the molecular cascades controlling neuronal circuit formation, we combine techniques such as gain- and loss-of-function mouse genetics, light microscope imaging of fluorescently labelled neuronal subpopulations, electrophysiological analysis and gene expression profiling.

Target-induced transcriptional control of dendritic patterning and connectivity in motor neurons by the ETS gene *Pea3*

The initial steps of sensory- and motor axon elaboration are controlled by cell-intrinsic factors and are determined even at stages before these neurons exit the cell cycle. Different classes of transcription factors control sequential steps in the hierarchy of differentiation of motor neurons in the developing spinal cord. However, our recent work has shown that in addition to specification of neurons by cell-intrinsic cues, signalling interactions with cues encountered by axons en route to the target or from the target region itself also have important roles in the

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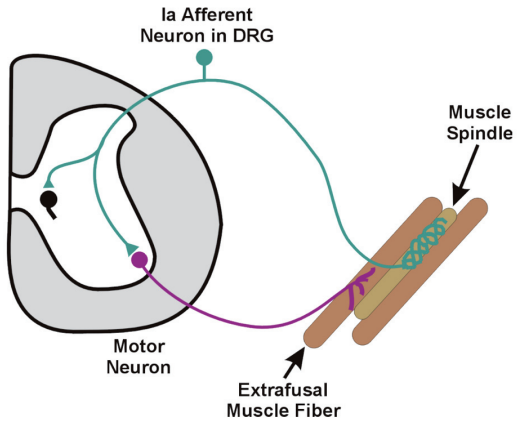

Fig. 1: Schematic representation of the mature spinal monosynaptic reflex circuit. Motor neurons project to extrafusal muscle fibers and receive synaptic input from group Ia afferent sensory neurons whose cell bodies reside in dorsal root ganglia (DRG).

specification of later steps of neuronal connectivity (reviewed by Ladle *et al*., 2007). We have identified two molecular pathways that link specific peripherally derived signals to the induction of the ETS transcription factors Pea3 and Er81 in defined subsets of motor neurons (Pea3) and DRG sensory neurons (Er81). Furthermore, we found that these retrograde signals from an intermediate target region act selectively to control the progressive specification and differentiation of distinct neuronal subpopulations through the activation of the ETS transcription factors Pea3 and Er81 (reviewed by Ladle *et al*., 2007).

We have now addressed whether there is a link between motor neuron dendrite orientation and patterns of connectivity, and we have also examined transcriptional programs regulating these processes (Vrieseling and Arber, 2006). Using anatomical, physiological and genetic strategies in the mouse, we have explored whether and how different motor neuron pools acquire selective presynaptic sensory inputs through the elaboration of distinct dendritic trees. We found that different motor neuron pools in the cervical spinal cord establish highly selective dendrite patterns covering distinct areas of the spinal cord. We found these patterns to be predictive of the responses that can be elicited in these motor neurons by sensory stimulation. Motor neuron pools elaborating radial dendrites receive monosynaptic input from group Ia proprioceptive afferents, whereas motor neuron pools not extending their dendrites into the central grey matter do not receive direct group Ia afferent input. Interestingly, we found that the initial establishment of motor neuron pool specific dendrite patterns is not dependent on the presence of proprioceptive afferents. In contrast, the expression of the ETS transcription factor *Pea3* in two cervical motor neuron pools is essential to control dendritic patterning and selectivity of sensory-motor connections (Figure 2). Since the induction of *Pea3* in these motor neuron pools is regulated by peripheral GDNF in a retrograde fashion, these findings suggest that target-derived signals exert profound input on the regulation of selectivity of presynaptic inputs and can thus act at a distance to control circuit specificity and functionality through the induction of transcriptional programs specifically expressed in distinct neuronal subpopulations.

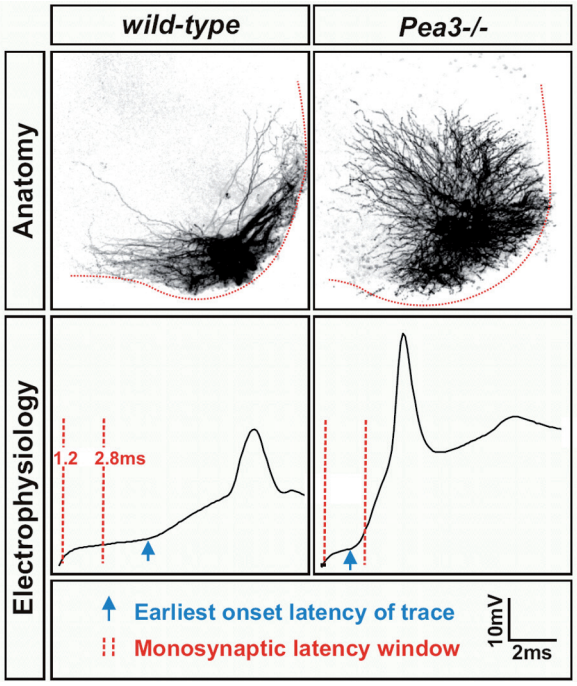

Fig. 2: ETS gene Pea3 is required for motor neuron dendrite patterning and sensory-motor connectivity. Figure displays dendrites of cutaneous maximus (CM) motor neurons in wild-type and Pea3 mutant mice (top). Functional consequences of Pea3 mutation on sensory-motor connections are shown by electrophysiology (bottom). Note that CM motor neurons in wild-type do not receive sensory input at monosynaptic latency, but Pea3 mutant CM motor neurons do. For details see Vrieseling and Arber, 2006.

Runx Transcription Factors and DRG Neuron Specification

Neuronal differentiation involves the acquisition of many specialized molecular properties that are essential for later neuronal function. Few aspects of the differentiated neuronal phenotype have a

greater functional impact than the expression of neurotrophic factor receptors. At different developmental stages, the precision of neurotrophic factor receptor expression determines the survival of neurons, axonal branching patterns, and the strength of synaptic connections. Yet the mechanisms that establish the selectivity of neurotrophic factor receptor expression by specific populations of vertebrate neurons remain obscure. In this study we have examined how different functional subpopulations of primary sensory neurons in the DRG acquire selective profiles of neurotrophic factor receptor expression that ensure the maturation of distinct sensory neuron subtypes.

In a screen to identify genes selectively expressed by functionally distinct subpopulations of DRG neurons, we identified two members of the Runx family of transcription factors to mark TrkC+ proprioceptive afferents (Runx3) and TrkA+ cutaneous afferents (Runx1) respectively. In a recent study, we have examined the mechanisms by which Runx transcription factor signaling contributes to the acquisition of defined subtype-specific DRG neuronal characters (Kramer *et al*., 2006). Using gain and loss of function genetic manipulations in the mouse, we show that Runx transcription factor signaling acts to subdivide broad subtypes of DRG neurons into distinct neuronal populations. Runx3 acts to diversify an Ngn1-independent neuronal cohort, and Runx1 controls neuronal diversification within Ngn1-dependent TrkA+ neurons. Specifically, Runx3 contributes to the differentiation of a DRG neuron subpopulation that expresses TrkC alone through selective erosion of TrkB expression within TrkC+ sensory neurons. Within TrkA+ cutaneous neurons, Runx1 suppresses the emergence of a CGRP peptidergic character and regulates the pattern of laminar termination in the dorsal spinal cord. Moreover, Runx3 and Runx1 activities are interchangeable with respect to the acquisition of these subtype-specific sensory neuron traits. Together, our findings suggest that Runx3 and Runx1 regulate the emergence of subpopulation-specific DRG neuron characters.

Our findings therefore provide evidence that Runx transcription factor signaling controls three critical elements of DRG neuronal phenotype: neurotrophin sensitivity, peptidergic neurotransmitter profile and axonal targeting. Thus Runx proteins serve as key transcriptional intermediaries in the assignment of the functional features of DRG sensory neuron subclasses that underlie the early steps of somatosensory processing (Kramer *et al*. 2006).

ETS transcription factor *Erm* controls subsynaptic gene expression in skeletal muscles

The alignment of pre- and postsynaptic elements of chemical synapses represents an important step in wiring neuronal circuits for function. This process involves the spatially coordinated enrichment and assembly of presynaptic neurotransmitter release machinery and postsynaptic structures at nascent synaptic sites. Despite the recognized importance of subcellular protein accumulation to synaptic sites, regulatory mechanisms orchestrating local accumulation of synaptic proteins to these highly specialized cellular compartments remain poorly defined.

At the neuromuscular junction (NMJ), subsynaptic nuclei underlie motor axon terminals within extrafusal muscle fibers and are transcriptionally distinct from neighboring nuclei. In this study, we show that expression of the ETS transcription factor *Erm* is highly concentrated at subsynaptic nuclei, and its mutation in mice leads to severe downregulation of many genes with normally enriched subsynaptic expression. *Erm* mutant mice display an expansion of the muscle central domain in which acetylcholine receptor (AChR) clusters accumulate, show gradual fragmentation of AChR clusters (Figure 3) and exhibit symptoms of muscle weakness mimicking congenital myasthenic syndrome (CMS).

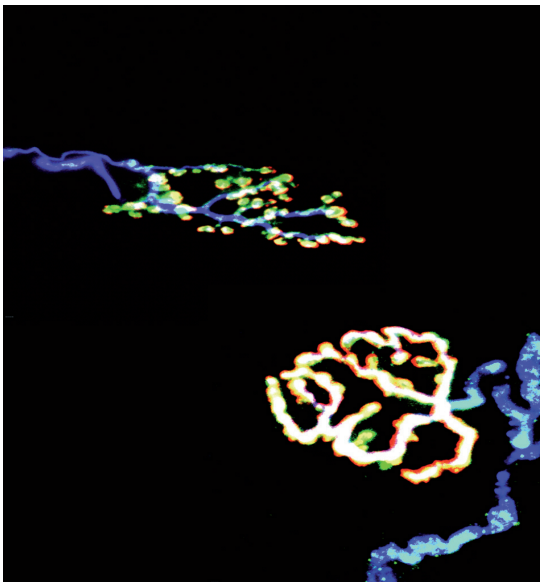

Fig. 3: Mutation of Erm leads to fragmentation of AChR clusters in alignment with innervating motor nerve terminals at neuromuscular junctions. Image shows close-up of a normal neuromuscular junction (bottom) and a neuromuscular junction in the absence of Erm (top). For details see Hippenmeyer et al., 2007

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Moreover, *Erm* mutation also leads to functional neurotransmission defects detected by electrophysiology. Together, our findings define *Erm* as an upstream regulator of a transcriptional program selective to subsynaptic nuclei at the NMJ and underscore the importance of transcriptional control of local synaptic protein accumulation.

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Stem Cells and Neural Development

Scope of research

Our group investigates the molecular pathways controlling neuronal development in higher vertebrates. To better understand mechanisms relevant to the development and diseases of the nervous system, we focus much of our work on neurotrophins and their receptors, as well as on cellular assays using neurons derived from embryonic stem (ES) cells.

Neurotrophins

In mammals, the 4 members of the neurotrophin gene family are involved in virtually all aspects of the biology of neurons. These include the control of cell survival and of axonal elongation, as well as neurotransmission and activity-dependent modification of synaptic function. Because of the possible roles of this signalling system in the function and dysfunction of the adult CNS, there is an increasing need to generate suitable models to study the role and mode of action of neurotrophins and of their receptors. The neurotrophin receptor $p75^{NTR}$ is mostly expressed during development and plays significant roles in inhibiting axonal elongation and in causing cell death This receptor is re-expressed in pathological situations such as axotomy, brain ischemia and epilepsy. With regard to the neurotrophin brain-derived neurotrophic factor (BDNF), its levels of expression and of secretion have been linked with complex behaviour in humans, including the regulation of food intake (work in collaboration with Karl Hofbauer), depression, episodic memory and anxiety. To explore its role in the adult, we generated mice that can live for several months while essentially lacking BDNF in the CNS following the selective deletion of the gene in neurons. These mice helped us to better understand the role of BDNF in various brain areas, including the striatum in particular. The biosynthesis, storage and secretion of endogenous BDNF, as well as it localisation in differentiated neurons, are also at the centre of our current studies.

Embryonic Stem Cells

We use extensively mouse embryonic stem (ES) cells to complement with cellular assays our *in vivo* research. While ES cells have an unlimited potential to divide they can also be selectively guided towards specific differentiation pathways. We found that mouse ES cells can generate essentially uniform populations of neurons that use glutamate as neurotransmitter. This was made possible following the observation that ES cells can be directed to form a virtually pure population of progenitors, namely Pax6-positive radial glial cells. Whether or not similar

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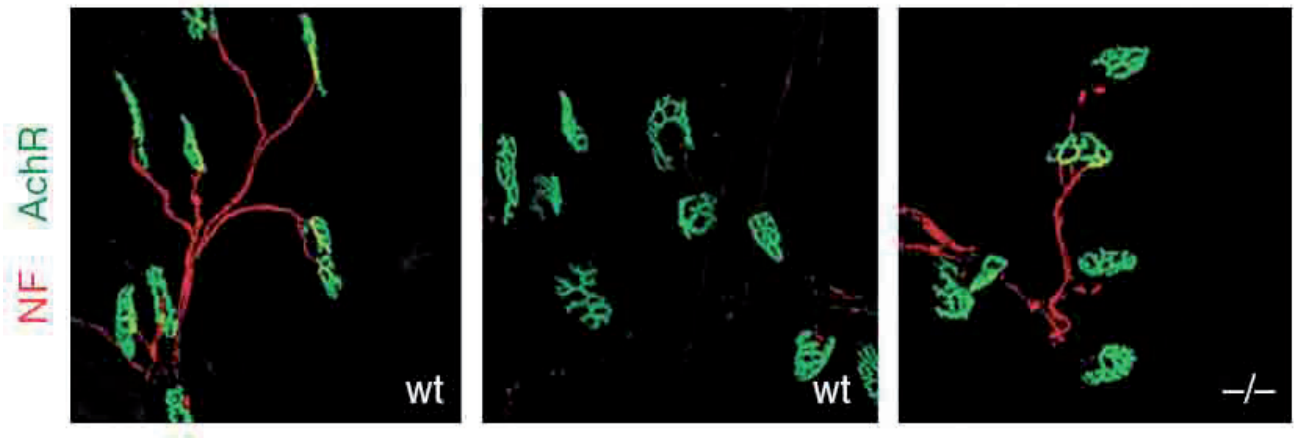
results can be obtained with human ES cells is also currently being explored. Mouse ES cells are now extensively used to study the impact of genes thought to be important for the development and function of the CNS, including in particular the transcription factor Pax6 and the DNA binding protein MeCP2. In collaboration with the group of Magdalena Götz (GSF; Neuherberg, Germany), we found that ES cells lacking both functional copies of Pax6 generate misspecified progenitors that go on to produce neurons synthezing the inhibitory neurotransmitter GABA, as opposed to excitatory neurotransmitter glutamate. These misspecified cells were also found to over-express $p75^{NTR}$ and to rapidly die *in vitro*. These observations could be verified in the developing cortex of pax6 mutant animals, thus indicating that the ES cell differentiation system leads to useful predictions. In the case of Pax6, it helped redefining the role of this transcription factor in the developing cortex and we now also use it in the context of mutations affecting MeCP2, a gene responsible for most cases of Rett Syndrome. Our hope is that the homogeneity and synchrony our in vitro differentiation system will help us identifying changes in gene expression that could explain altered electrophysiological properties of neurons lacking MeCP2 (work performed in collaboration with Kaspar Vogt).

The ES cell-based system also allowed to generate a new *in vitro* model of axonal degeneration by using cells over-expressing of $p75^{NTR}$ in a controlled fashion. In collaboration with the proteomic group at Novartis, we identified by mass spectrometry Galectin-1 as a protein regulated by $p75^{NTR}$ and causally involved in the degeneration of processes. The role of this endogenous lection was also studied in mouse mutants lacking the corresponding gene and explored at the neuro-muscular junction in collaboration with Shuo Lin and Markus Rüegg (see illustration below).



Left pannel (wt) Axon terminals, visualized with antibodies to neurofilaments NF, innervate the neuro-muscular junction, revealed with fluorescent alpha bungaortoxin that stains post-synaptic acetylcholine receptors, AchR. While in wild-type animals, axons rapidly degenerate following section of the sciatic nerve (middle panel) many survive 24 h after nerve section in animals lacking Galectin-1 (-/- panel). This work also showed that following axotomy p75NTR and Galectin-1 are both rapidly up-regulated in Schwann cells distal to the lesion (for additional details, see Plachta et al., 2007).

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New pharmacological concepts for the treatment of obesity and cachexia

During the reporting period 2006/2007 our research focussed on the validation of new concepts for the treatment of obesity or cachexia. Obesity shows a prevalence of more than 30% in the USA and other industrialized countries and has become a serious worldwide health problem. Despite an ever increasing number of potential pharmacological targets drug development in this indication has not been as productive as expected. No more than three drugs are currently available for the long-term treatment of obesity and they have only moderate efficacy. Cachexia is a syndrome which consists of a reduced appetite and a loss of fat and skeletal muscle mass. It is usually associated with chronic diseases such as cancer, infection or inflammation and is recognized as an independent risk factor for morbidity and mortality. Cachexia cannot be adequately treated by nutritional support and no effective pharmacological agents are known.

We chose the melanocortin-4 receptor (MC4R) as a molecular target for both indications because it plays an important role in the regulation of appetite and autonomic function (for details see Figure 1). Our pharmacological approach consisted in the generation and characterization of functionally active antibodies against extracellular sequences of the MC4R. Our initial studies in rats revealed an obese phenotype after immunization against the N-terminal domain of the MC4R. These experiments are described in more detail in our previous biennial report and have been published in 2007.

In subsequent experiments we attempted to generate monoclonal anti-MC4R antibodies since such molecules can be produced in unlimited amounts. Immunization of mice against the N-terminal domain resulted in a monoclonal antibody which blocked the activity of the MC4R. In further in vitro studies it was characterised as an inverse agonist with non-competitive antagonistic activity. A priority claim has been filed in collaboration with the Technology Transfer Unit of the University of Basel. The monoclonal antibody is currently being produced in larger amounts for acute and chronic studies on its blood-brain penetration after peripheral administration. Thereafter it will be used as a pharmacological tool to explore the therapeutic potential of MC4R blockade in various disease models. A simple model for cachexia-induced anorexia, i.e. the loss of appetite in association with inflammatory diseases, has been established and validated in our laboratory. It consists of the continuous monitoring of food intake in rats after the acute administration of lipopolysaccharide.

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Our immunization studies on the MC4R in rats are being complemented with several series of immunizations against extracellular sequences of the MC3R. These experiments were initiated because presynaptic MC3Rs appears to modulate the release of the MC4R agonist alpha-melanocyte stimulating hormone (alpha-MSH) from nerve terminals. However, the precise function of the MC3R is not known because of the lack of suitable experimental tools. Based on preliminary results with two antigens from the first and the third extracellular loop a long-term immunization study has been started in rats which are chronically instrumented with telemetry transmitters. This makes it possible to monitor not only endocrine and metabolic parameters but also blood pressure, heart rate, body temperature and locomotor activity.

Our observation that immunization against the MC4R resulted in an obese phenotype in rats prompted us to explore whether comparable findings can be obtained in obese patients. We therefore initiated a clinical study in collaboration with the University Clinic in Strasbourg, France, and the communal hospital in Baden-Baden, Germany. These institutions provided more than 200 plasma samples from patients with a wide range of body mass indices (BMI). The plasma samples were screened in our laboratory with an ELISA assay using the N-terminal peptide sequence of the MC4R. Positive samples were further assessed by flow cytometry (FACS) and cAMP measurements in HEK293 cells which overexpressed the human MC4R.

The presence of auto-antibodies against the MC4R in humans and the increased prevalence of such autoantibodies in patients with increased BMI would suggest that they might play a pathogenetic role in the development of obesity. By using such a test in the clinic a subgroup of obese patients with a common pathophysiology could be identified and possibly causally treated. Several options for further clinical studies are currently being explored and experimental studies on possible therapeutic approaches have been initiated in our group.

Our studies on the role of brain derived neurotrophic factor (BDNF) as a downstream mediator of the MC4R have been completed and are subject of a recent publication. In this paper we reported for the first time that MC4R stimulation in isolated hypothalami *in vitro* induced a release of BDNF into the incubation medium. In experiments *in vivo* we have shown that BDNF and a MC4R agonist have comparable effects on food intake. Moreover, the effects of MC4R stimulation could be prevented by the prior administration of a selective anti-BDNF antibody. In an additional series of experiments in rats with telemetry transmitters we could demonstrate that BDNF and a MC4R agonist also showed comparable cardiovascular effects, i.e. both agents induced an increase in blood pressure and heart rate which was associated with a rise in body temperature. These effects of MC4R stimulation could also be prevented by the anti-BDNF antibody. Thus BDNF appears to mediate not only the anorectic but also the autonomic effects of MC4R stimulation. This project was performed in collaboration with Prof. Yves Barde who also supplied the monoclonal antibody against BDNF.

Karl G. Hofbauer contributed to pharmacology teaching in the Medical Faculty and the Faculty for Natural Sciences at the University of Basel. He took on responsibility for the planning and coordination of pharmacology teaching for medical students in the new Bologna system up to the Bachelor level. He contributed to a newly established course for a Master of Advanced Studies in Pharmacology degree at the University of Basel. Karl G. Hofbauer gave also lectures on drug discovery and development at the University of Zuerich.

Karl Hofbauer was involved in several continuing education activities including the co-organization of and the active contribution to the annual “Cardiovascular Pharmacology Seminar” in Luzern. Together with several clinical colleagues Karl G. Hofbauer continued to co-organize a lecture series at the University of Basel with focus on obesity, metabolism and nutrition (OMeN).

Karl G. Hofbauer and members of his group presented their findings at several national and international scientific meetings including an invited lecture at the Joint Meeting of the Swiss and Canadian Pharmacological Societies in Banff, Canada, in early 2007.

In addition to original papers and book chapters two reviews from our group covered the recent developments in the field of anti-obesity drugs.

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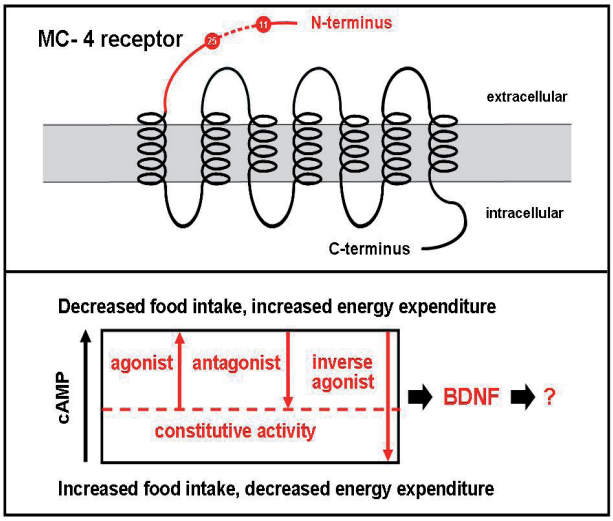

Figure 1: In the upper panel a schematic illustration of the structure of the MC4R is given. This receptor is a member of the 7-transmembrane domain receptor family. From studies in rodents and from mutations in humans information about the functional importance of single amino acids and peptide domains became available. The N-terminus is thought to interact with other extracellular domains to maintain the constitutive activity of this receptor. It was therefore chosen as the main target for our immunization experiments and for our clinical studies on auto-antibodies in patients with obesity.

In the lower panel the functional states of the MC4R are schematically illustrated. The MC4R is coupled to adenylyl cyclase via a Gs protein. Thus the activity of this receptor can be determined by measuring the rate of cAMP production. Under basal conditions the receptor shows constitutive activity. The endogenous agonist is alpha-melanocyte stimulating hormone (alpha-MSH), a post-translational breakdown product of pro-opiomelanocortin (POMC). The MC4R is unique in having an endogenous inverse agonist, Agouti-related protein (AgRP). Pharmacological blockade of the receptor can also be induced by synthetic antagonists of alpha-MSH or by antibodies directed against the N-terminal sequence of the MC4R which act as inverse agonists. It is likely that inverse agonists have a higher efficacy than antagonists because they not only reverse the activation of the receptor by an agonist but also reduce its constitutive activity.

The activation of the MC4R mediates a decrease in food intake and an increase in energy expenditure. Thus MC4R agonists could be useful drugs for the treatment of obesity. However, the MC4R has not only effects on energy balance but also influences autonomic function. MC4R activation results for instance in an increase in blood pressure and heart rate. In order to generate agonists with a selective effect on energy balance it is essential to identify downstream mechanisms which may mediate the MC4R induced effects separately. BDNF appears to be such a downstream mechanism of the MC4R but it shows no selectivity for the metabolic and autonomic effects. The subsequent steps in the MC4R downstream pathway are still unknown.

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Towards the roles of sleep in neuronal functions

Sleep is operationally defined as a natural state of decreased voluntary motor activity, reduced response to stimulation, and stereotypic posture. Yet, the unambiguous hallmarks of the sleeping state are generated by the brain. We would like to know: Which ionic and signalling events are specific for a sleeping neuron, how do they contribute to the sleep state and why is sleep beneficial for neuronal functions?

Sleep is a behavioral state created by the physiology of neurons and neuronal networks, interconnected over distances of millimeters. To understand sleep, we need to combine behavioral manipulations and observations with cellular recording and long-range fiber tracing techniques.

Here are some examples of our research.

Insufficient sleep reversibly alters bidirectional synaptic plasticity and NMDA receptor function (C. Kopp, F. Longordo)

Insufficient sleep impairs cognitive functions in humans and animals. However, whether long-term synaptic plasticity, a cellular substrate of learning and memory, is compromised by sleep loss *per se* remains unclear, due to confounding factors related to sleep deprivation (SD) procedures in rodents. Using an *ex vivo* approach in mice, we show that sleep loss rapidly and reversibly alters bidirectional synaptic plasticity in the CA1 area of hippocampus. A brief (~4 hrs) total SD, respecting the temporal parameters of sleep regulation and maintaining unaltered low corticosterone levels, shifted the modification threshold for long-term depression/potentiation (LTD/LTP) along the stimulation frequency axis (1-100 Hz) towards the right. Reducing exposure to sensory stimuli by whisker-trimming did not affect the SD-induced changes in synaptic plasticity. Recovery sleep reversed the effects induced by SD. An alteration in the molecular composition of synaptic N-methyl-D-aspartate receptors (NMDARs) towards a greater NR2A/NR2B ratio accompanied the effects of SD and was reversed after recovery sleep.

We recently tested whether and how SD-induced alteration at the level of synaptic plasticity and receptor expression are causally linked. Using NMDAR-subtype-specific pharmacological agents, we found that the 5 Hz-induced LTD after SD was NMDAR-dependent and specifically required the activation of NR2A-NMDAR subtypes. To determine the role of NR2A-NMDARs in the induction of synaptic plastic-

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ity, we monitored the temporal summation of synaptic NMDAR-currents in response to 5 Hz stimulation trains. Pharmacological and biophysical analysis of these summated currents revealed that the contribution of the NR2A-NMDARs was favored. Notably, the enhanced contribution manifested as a more rapidly decaying tail current at the end of the train, suggesting that an enhanced NR2A-content of NMDARs translates into how CA1 synapses temporally integrate repeated stimulation. By using an unparalleled, particularly mild form of SD, these studies describe a novel approach towards dissociating the consequences of insufficient sleep on synaptic plasticity from non-specific effects accompanying SD in rodents. We establish a framework for cellular models of cognitive impairment related to sleep loss, a major problem in modern society.

Ca^{2+} entry through T-type Ca^{2+} channels gates sleep-related oscillations through competing targets in thalamic dendrites (L. Cueni)

Neurons in the thalamocortical system co-operate to produce synchronized, rhythmic network activity that underlies slow waves characteristic of sleep electroencephalograms (EEGs). Rhythmogenesis is accompanied by low-threshold burst discharges in thalamic neurons, which are carried by low-voltage-activated, T-type Ca^{2+} currents. Although Ca^{2+} ions entering through T-type Ca^{2+} currents are the electrical charge carriers underlying low-threshold bursts, the associated intracellular Ca^{2+} ($[Ca^{2+}]_i$) dynamics and their role in sleep physiology remain largely unknown. We hypothesized that targets selectively regulated by T-type Ca^{2+} currents would be important for controlling sleep-related cellular oscillations and could have implications for sleep physiology. We focused on the nucleus reticularis thalami (nRt), a thin inhibitory network located strategically within thalamocortical networks to control information flow. Prominent forms of rhythmic bursting in the nRt accompany the major forms of low-frequency EEG oscillations typical for sleep. We show that in the dendrites of nRt, $[Ca^{2+}]_i$ increases are dominated by T-type Ca^{2+} currents and shape rhythmic bursting by creating a competition between Ca^{2+}-dependent SK-type K^+ currents and Ca^{2+} uptake pumps. Via selective activation of dendritically located SK2 channels, oscillatory bursting is generated along major dendritic segments. The sequestration of Ca^{2+} by sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), together with cumulative T-type Ca^{2+} channel inactivation, antagonizes SK2 channel activation and dampens oscillations. Mice lacking the SK2 channel gene demonstrate a >3-fold reduction in low-frequency rhythms in the EEG of non-rapid-eye-movement sleep. The interplay of T-type Ca^{2+} channels, SK2 channels, and SERCAs in nRt dendrites comprises a specialized organization handling Ca^{2+} entry through T-type Ca^{2+} channels to regulate oscillatory dynamics related to sleep. We think that assessing the roles of the T-type Ca^{2+}/SK2 channel/SERCA triad in thalamocortical networks could help to identify targets for improving NREMS continuity and/or depth, in disorders in which NREMS quality is impacted such as in primary insomnia or insomnias associated with psychiatric or neurological disorders.

Powerful inhibitory control of higher-order thalamic nuclei by giant-sized synaptic terminals from anterior pretectum (N. Wanaverbecq)

All thalamic nuclei are controlled by strong inhibitory afferents of intrathalamic origin. This inhibition via nRt determines thalamic functions related to the gating of sensory input and to the generation of sleep rhythms. A large region of the thalamus, however, the so-called higher-order nuclei (HOn), is innervated by distinct inhibitory projections of extrathalamic origin. These arise in subcortical motor centers and render HOn largely non-responsive to sensory afferents, unless inhibition is relieved through top-down motor control. We recently demonstrated that HOn are selectively innervated by giant-sized inhibitory terminals arising from the anterior pretectum (APT). To assess the synaptic basis underlying the potent inhibition of HOn, we used electrophysiological methods to study quantal properties and short-term plasticity of APT-HOn synapses. We compared these to the characteristics of intrathalamic inhibitory synapses maintained simultaneously in slices of P28 rats. To make these long-range fiber tracts accessible for *in vitro* analysis, we developed a novel long-range fiber labeling method that is based on electroporation of dextran-coupled fluorescent dyes (Figure 1). Multiple probability fluctuation analysis showed that unitary APT-HOn afferents contained a greater number of independent release sites (~12) compared to intrathalamic inputs (~5), while quantal size and release probabilities were similar. Electron microscopic analysis revealed giant-sized boutons of APT fibers containing multiple active zones (~12 per bouton), whereas intrathalamic projections formed several boutons with a single active zone. Brief stimulation trains (10 - 100 Hz) to APT afferents produced synaptic responses with a pronounced persisting component, whereas intrathalamically generated currents decayed completely. The assembly of multiple active zones into one synaptic terminal, while leav-

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ing them functionally independent, could represent a device to ensure powerful inhibitory control over a large range of presynaptic activities.

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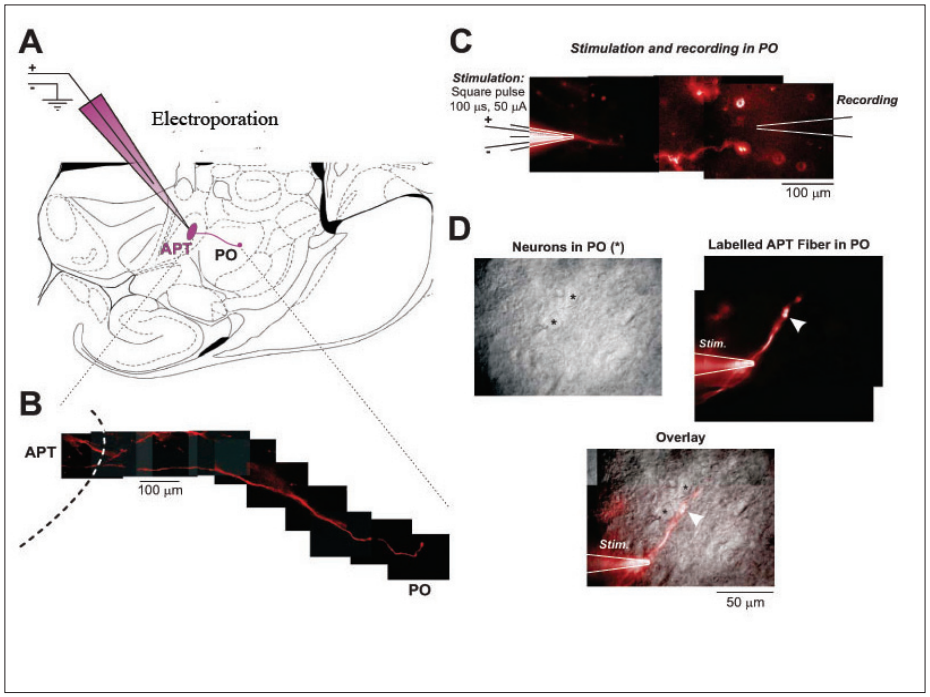

*Figure 1: Fluorescent labeling of individual long-range projections acutely in a brain slice, in combination with electrophysiological recording. A Slice prepatation showing location of the brain nuclei called APT and PO (see text for further details), along with the electroporation electrode. B Single axonal fiber arising in APT and projecting to PO. C Position of stimulation and recording electrodes D Identification of neurons in the vicinity of labeled fibers (labelled with *) using differential interference contrast microscopy (left, top) combined with fluorescence microscopy (right, top). Overlay (bottom) shows close colocalization of cell bodies with labelled fibers (arrowhead).*

Construction of the brain: a molecular genetic analysis of brain development in Drosophila

The brain is the most complex organ in all higher organisms and is characterized by an enormous diversity and specificity of neurons that are interconnected via complex circuitry. Understanding the organizing principles that underlie the complex architecture of the brain remains one of the most challenging problems of modern biology. The extremely high degree of anatomical complexity of the mature brain arises progressively during its development from the neural epithelium. Neural stem cell-like progenitor cells in the regionalized neuroectoderm become specified, initiate proliferation and generate neural progeny that segregate into distinct regional collectives of neurons which become fully functional through process outgrowth and the establishment of synaptic connections.

Recent advances in molecular and developmental neurobiology of several neurogenetic model systems have resulted in the identification and characterization of a number genes involved in neural regionalization, specification, proliferation, axonal pathfinding and synaptogenesis. However, it is still largely unclear how these genes and the developmental processes that they control, relate to the complex architecture and organization of the adult brain. A neurogenetic model system that is ideal to analyse the construction principles of the brain is Drosophila. The Drosophila brain is a highly complex structure composed of hundreds of thousands of neurons that are interconnected in exquisitely organized neuropil structures such as the mushroom bodies, central complex, antennal lobes, and other specialized neuropil areas as well as major fiber tracts. Moreover, in Drosophila powerful classical genetic, molecular genetic, genomic and transgenic technologies for cellular manipulation, visualization of gene expression, insertional mutagenesis, gene knockdown, and targeted gene misexpression are available. Recently, significant advances have been made in analyzing brain development in Drosophila. The work of our lab has contributed to these advances in four areas.

Conserved genetic mechanisms of early regionalization

In recent years, we have studied the role of key high-order developmental control genes in patterning and regionalization of the Drosophila brain. The transcription factor encoding genes acting in anteroposterior patterning include the *otd/Otx* and *ems/Emx* genes which are involved in specification and regionalization of the anterior brain, Hox genes which are involved in the differentiation of the posterior brain, and *Pax* genes which are involved in the

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development of the anterior/posterior brain boundary zone. Transcription factor encoding genes acting in dorsoventral pattering are the *vnd/Nkx*, *ind/Gsh* and *msh/Msx* genes which are involved in specifi-

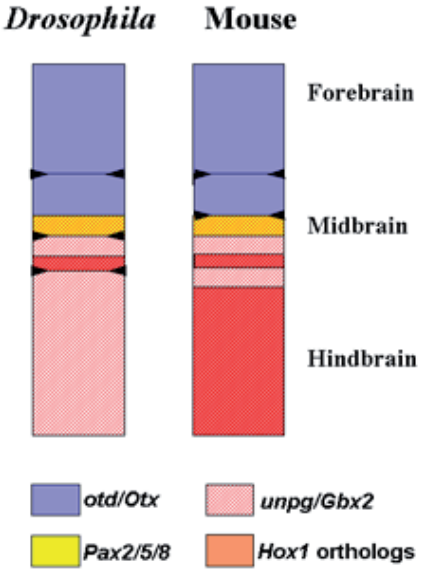

Fig. 1: Conserved anteroposterior pattern of developmental gene expression in embryonic brain development. Simplified schematic diagram of otd/Otx, Pax2/5/8, unpg/Gbx and Hox gene expression in the embryonic CNS of Drosophila (left) and mouse (right). Expression domains are color coded; anterior is to the top. For details see Hirth and Reichert, 2007.

cation of columnar neurogenic arrays in the brain. Our work in Drosophila, together with work of others on the development of the mammalian brain, provides evidence that all of these gene sets have evolutionarily conserved roles in brain development, revealing a surprising conservation in the molecular mechanisms of brain development. Our most recent findings as well as reviews of their implications for brain evolution are in Sprecher *et al*. (2007), Meier *et al*. (2006), Lichtneckert and Reichert (2007), and Hirth and Reichert (2007).

Polycomb group genes and neural stem cell proliferation

The complex circuitry of the brain derives from a highly restricted set of approximately 200 neural stem cell-like progenitors called neuroblasts. The controlled proliferation of these neuroblasts is essential for generating the appropriate number of neurons in brain circuitry. In a lineage-based analysis of Drosophila brain development, we have uncovered a key role of the epigenetically acting *Polycomb group* genes in neuroblast survival and proliferation. Our study shows that proliferation in neuroblast lineages is dramatically reduced in the absence of the genes *Polycomb, Sex combs extra, Sex combs missing, Enhancer-of-zeste (E(z)*, or *Supressor of zeste 12*. This is due to the fact that loss of any of these *Polycomb* group genes leads to aberrant de-repression of posterior *Hox* gene expression in neuroblasts, which in turn causes premature neuroblast death and early termination of proliferation in the mutant clones. These findings demonstrate that *Polycomb* group genes are essential for normal neuroblast survival and proliferation in the Drosophila brain and imply that repression of aberrant reactivation of *Hox* genes may be a general and evolutionarily conserved role for *Polycomb group* genes in brain development (Bello *et al*., 2007).

Loss of proliferation control and brain tumor formation

Whereas mutations in *Polycomb group* genes lead to underproliferation, mutations in other key control genes can lead to overproliferation and, hence, result in brain tumors. One of these is the tumor sup-

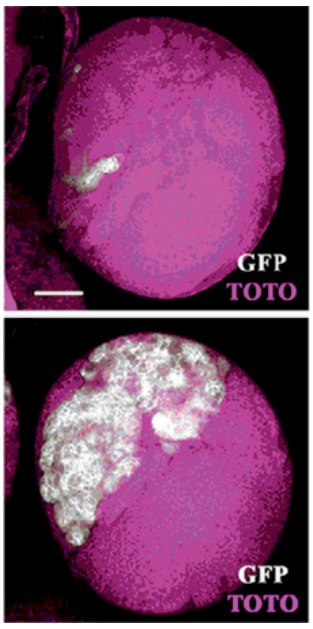

Fig. 2: Mutations in the brat gene result in tumorigenic overproliferation in the Drosophila brain. Single wildtype neural stem cell clones are highly restricted in a larval brain hemisphere (top). In contrast, neural stem cell clones that are mutant in the brat gene overproliferate and fill out large areas of a larval brain hemisphere (bottom). Neural stem cell clones are labeled with GFP using the mosaic analysis with a repressible cell marker (MARCM) technique. For details see Bello et al., 2006.

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pressor gene *brain tumor (brat)*. We have studied the role of *brat* in brain development and tumorigenesis using the powerful clonal labelling and mutational methods made possible by MARCM in combination with GAL4/UAS. Our findings demonstrate that *brat* negatively regulates cell proliferation during larval brain development and identify the cell fate determinant *prospero* as a key downstream effector of *brat* in cell fate specification and proliferation control. In consequence, loss of *brat* in a single brain neuroblast clone leads to uncontrolled proliferation which persists into adulthood and results in a lethal brain tumor. Moreover, *prospero* mutant clones phenocopy *brat* mutant clones in the larval central brain, and targeted expression of wild-type *prospero* in *brat* mutant clones results in rescued clones and prevents brain tumor formation. These studies provide detailed insight into the molecular mechanisms which link loss of neural stem cell proliferation control to brain tumor formation (Bello *et al*., 2006).

Lineage-based organization of complex brain architecture

In Drosophila each brain neuroblast gives rise to a lineally-related set of neurons and glia ("neuroblast clones") which generally correspond to distinct architectural modules of the brain. To analyse the role of developmental control genes in determining the anatomical features of neuroblast clones, we studied the homeodomain transcription factor-encoding *ems* gene. The *ems (empty spiracles)* gene is a member of the cephalic gap gene family that acts in specification of the anterior embryonic neuroectoderm. Our work shows that *ems* is also expressed in the mature brain in the lineage-restricted clonal progeny of a single neuroblast in each brain hemisphere. These *ems*-expressing neuronal cells are located ventral to the antennal lobes and project a fascicle to the superior medial protocerebrum. All neurons in this lineage persistently express *ems* during postembryonic development and in the adult. Mosaic-based mutant analysis and genetic rescue experiments demonstrate that *ems* function is autonomously required for the correct number of cells and the formation of the correct projections in this specific lineage. This analysis of *ems* expression and function reveals novel roles of a cephalic gap gene in translating lineage information into cell number control and projection specificity in an individual clonal unit of the brain (Lichtneckert *et al*., 2007).

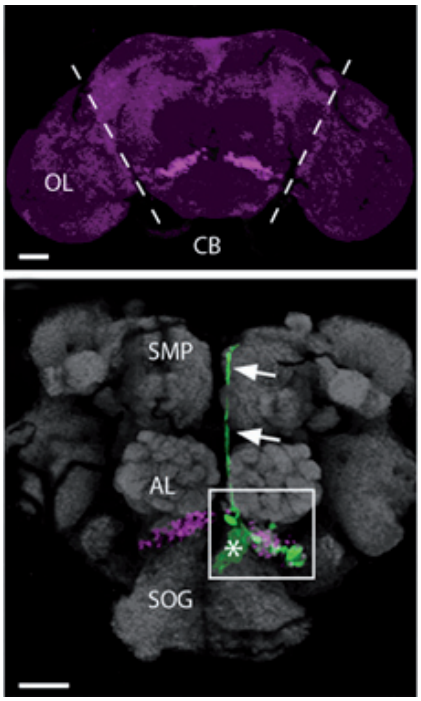

Fig. 3: The ems gene is expressed in two bilaterally symmetrical clusters of lineally related neurons in the adult Drosopila brain. Anti-Ems immunostaining reveals the cell bodies of the two ems-expressing cell clusters (top). Combination of MARCM-based clonal labelling technique and anti-Ems immunostaining demonstrates that the ems-expressing cells in a cluster are lineally related and derive from a single neural stem cell (bottom). For details see Lichtneckert and Reichert, 2007.

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Molecular mechanisms involved in synapse formation and neuromuscular disease

The main goal of our research is the understanding of the molecular pathways involved in the formation, the function and dysfunction of synapses. These questions are studied at nerve-muscle synapses (Fig. 1A) and at synapses between neurons in the central nervous system (Fig. 1B). The understanding of such molecular mechanisms serves us also as basis to investigate new ways of interfering with diseases in which synaptic function is impaired. In particular, we focus on neuromuscular diseases.

The MAP kinase pathway is involved in the development of the neuromuscular junction

Because of its large size and good accessibility, the synapse between motor neurons and skeletal muscle fibers, called neuromuscular junction (NMJ), is the best studied synapse (see Fig. 1A). Experiments over the last two decades have provided compelling evidence that the formation of the postsynaptic structure at the NMJ depends on at least 5 different molecules: (1) The nerve-released extracellular matrix agrin; (2) the receptor tyrosine kinase (MuSK) that is activated by agrin; (3) the intracellular adaptor molecule (DOK7), which binds to activated MuSK; (4) the acetylcholine receptor (AChR) subunits; and (5) the AChR adaptor molecule rapsyn.

However, still little is known of how agrin activates MuSK and which intracellular pathways are subsequently involved in the formation of the NMJ. In one project, we use gene profiling to identify genes whose expression is regulated during the formation of postsynaptic structures at the NMJ. To do this, we have used an experimental paradigm in which recombinant agrin protein is injected into adult muscle *in vivo*. As previously reported, this injection induces the formation of postsynaptic structures that are in all aspects identical to those induced by innervation. By comparing gene expression profiles in regions that contain many such agrin-induced postsynaptic structures with those of regions that are devoid of postsynapses, we found upregulation of genes that belong to the mitogen-activated protein (MAP) kinase pathway. Subsequent studies have shown that this pathway plays a crucial role in postsynaptic differentiation and that the MAP kinase phosphatase, MKP-1, is important to set up an auto-inhibitory feedback loop that contributes to the assembly of the postsynaptic apparatus. Moreover, we find that activation of the MAP kinase pathway by agrin-MuSK signaling is also necessary to activate local gene transcription in the myonuclei underlying the synapse. Interestingly, the same pathway has also been implicated in the brain to regulate synapse structure during the

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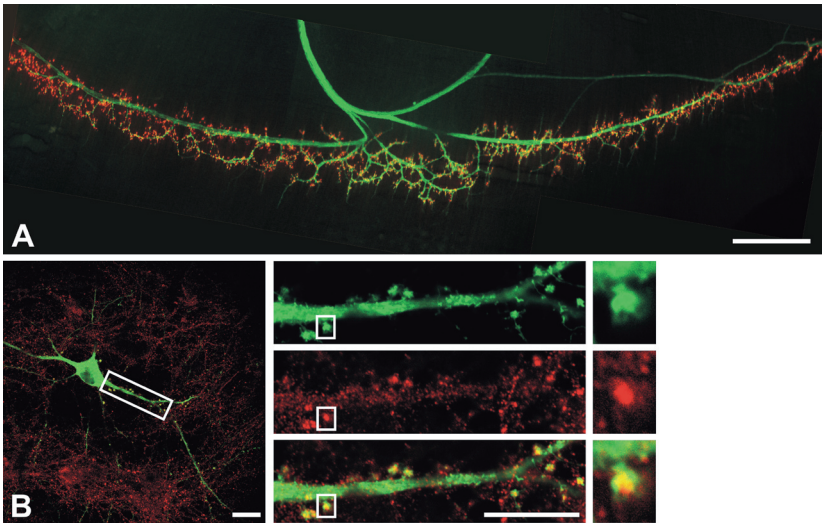

Figure 1: ***(A)****. Whole mount of mouse diaphragm at embryonic day 17.5. Motor nerve is visualized by transgenic expression of YFP (green), postsynaptic acetylcholine receptors are visualized with rhodamine-labeled α-bungarotoxin (red).* ***(B)****. Hippocampal neurons cultured for 14 days that were transfected with a GFP encoding plasmid (green) at day 7. Postsynaptic structures are visualized with an antibody to PSD 95 (red). Note that the postsynaptic neurotransmitter receptors are clustered in both types of synapses. Also note the big difference in size between the two types of synapses. Scale bars: 500 µm (A); 20 µm (B).*

process of learning and memory. Our work thus provides strong evidence that neuron-muscle and neuron-neuron synapses are not only similar in structure and function but also in the molecular mechanisms that establish these connections. In agreement with this conclusion, we have recently shown that excitatory synapses are malformed in the brain of agrin-deficient mice in which the perinatal death was rescued by the transgenic expression of agrin in motor neurons. Interestingly, this phenotype is also accompanied by changes in the MAP kinase pathway.

Experimental treatments of congenital muscular dystrophy

Neuromuscular diseases are rare genetic disorders characterized by a generalized and progressive loss of muscle mass that causes early morbidity and mortality. Although there has been tremendous progress in the understanding of the molecular mechanisms underlying these diseases, they are still orphan diseases and currently no curative treatments are available. Congenital muscular dystrophy (CMD) is a heterogeneous and clinically distinct group of muscular dystrophies with frequently early onset of symptoms. Laminin-α2 (merosin)-deficient CMD, called MDC1A, accounts for approximately 30 – 40% of all CMD patients. MDC1A is characterized by severe neonatal hypotonia associated with joint contracture, inability to stand or walk and death in early life. The disease is accompanied by a peripheral neuropathy originating from demyelination in the peripheral and central nervous system.

There is a well established mouse model, the *dy*^{W} mouse, which reiterates several key aspects of MDC1A pathology of human patients. Using these mice, we have shown that muscle-specific expression of a miniaturized form of the extracellular matrix molecule agrin or perlecan ameliorates disease progression. In another approach, others have shown that inhibition of apoptosis by transgenic overexpression of BCL2, or by deletion of the pro-apoptotic Bax gene, prolongs survival and ameliorates disease-specific parameters in the *dy*^{W} mouse. In collaboration with Santhera Pharmaceuticals Ltd, we have therefore investigated whether the pharmacological inhibitor of apoptosis, SNT-317 (Omigapil), ameliorates disease progression in the *dy*^{W} mouse. Indeed, substantial improvement was observed including a reduction of (1) early mortality, (2) loss of body weight, (3) skeletal abnormalities (Fig. 2) and (4) locomotory activity. We are currently investigating whether a combination of SNT-317 and mini-agrin shows any synergistic effect. In conclusion, these experiments have set the basis that may lead to new therapies for this orphan disease.

Copine family members regulate spine morphogenesis and synapse formation

Spines, which protrude from dendrites, are the principal site of excitatory synapses and structural changes of spines are thought to contribute to learning and memory. Several molecules have been identified as potential regulators of spine development and the function of these proteins is often regulated by activity-induced changes in intracellular calcium. The screening described above in which we identified genes that are transcriptionally regulated during the formation of postsynaptic structures in skeletal muscle, has also led to the identification of Copine 3.

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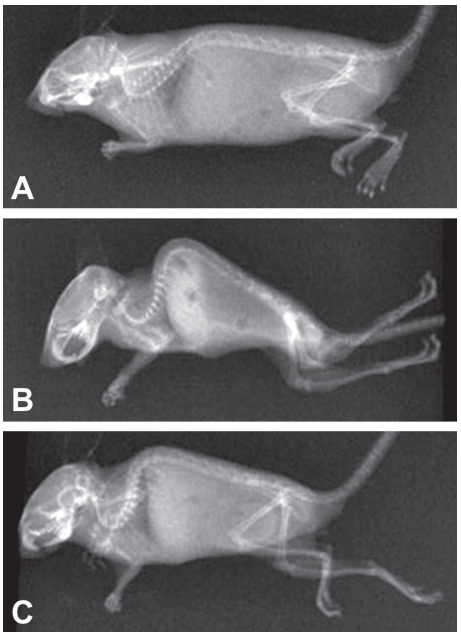

Figure 2: Effect of SNT-317 on vertical spine deformation (kyphosis) visualized by X-ray analysis. Pictures show eleven week-old wild-type (A), dy^W (B), and dy^W mice treated with SNT-317 (C). Treatment of dy^W mice with SNT-317 clearly ameliorates kyphosis.

Copine 3 belongs to a family of proteins that consists of at least 8 members in mammals. Copines are conserved from protozoa to human and are characterized by two highly conserved C2 domains, which are known to bind calcium, and an A domain thought to mediate interaction with other proteins.

We found that expression of several Copines is also upregulated during synapse formation between neurons. Analysis of the function of all Copine family members during synapse formation by RNA interference showed that the knockdown of Copine 3 reduced the formation of dendritic protrusions while a knockdown of Copine 6 triggered the formation of many, small spine-like structures on dendrites. Importantly, the density of synapses and the incorporation of AMPA receptors into postsynaptic spines was also increased. Copine 6 is enriched in spines and binds to Copine 3 and to synaptic membranes. Moreover, Copine 3 and Copine 6 interact with the small GTPase Rac1 and its effector protein Pak1. Thus, our studies are the first to characterize the Copines as a family of molecules that plays a role in the formation and maturation of synapses between neurons in the CNS.

The role of mTORC1 and mTORC2 in muscle and brain

Sizing of an organ during development or during adaptation in the adult can be controlled by alterations in either the number or the size of cells. These two mechanisms are fundamentally different from each other and require distinct sets of genes. Both, neurons and skeletal muscle fibers are postmitotic and thus changes in size are thought to be based mainly on cell growth and not on cell proliferation. The mammalian target of rapamycin (mTOR), which was identified in yeast by Michael Hall and collaborators at the Biozentrum, is known to be important for the regulation of organ and cell size. In collaboration with the laboratory of Michael Hall we have shown that mTOR, unlike previously assumed, assembles into two distinct protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), that can be distinguished by their sensitivity to rapamycin. The two complexes are also characterized by two distinctive binding proteins called raptor and rictor. To investigate the function of mTORC1 and mTORC2 in muscle and brain, we have generated conditional knock-out mice that lack raptor or rictor

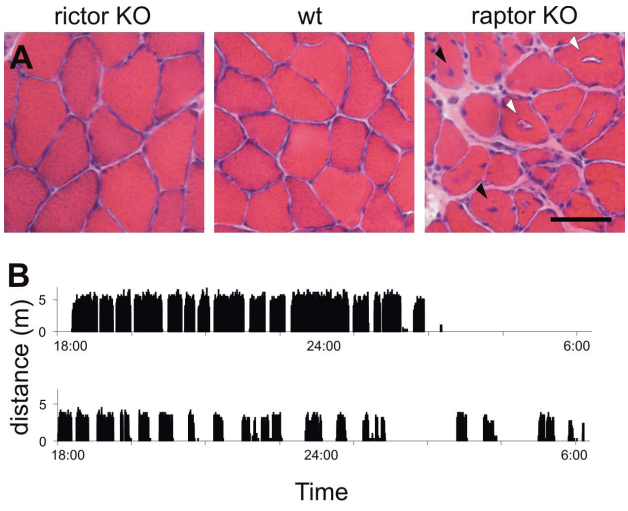

Figure 3: Characterization of mice that are deficient for rictor (rictor KO) or raptor (raptor KO) in skeletal muscle. ***(A)*** *Hematoxilin & eosin staining of soleus muscle from 140 day-old mice. No difference is observed in rictor KO mice. Raptor KO mice contain centralized nuclei (black arrowheads) and mononuclear cells indicative of muscle dystrophy. Furthermore central cores (white arrowheads) can be seen.* **(B)** *Representative activity pattern (10 sec per bin) of a wild-type mouse (upper panel) and a raptor KO mouse (lower panel). Raptor KO mice are more quickly exhausted and run less. Scale bar: 50 µm.*

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in skeletal muscle fibers (see below) and we are in the process of generating mice that are deficient of either of these proteins in the brain.

Several lines of evidence suggest an essential role of mTOR signaling in the control of skeletal muscle growth. In particular, activation of upstream components of mTOR, such as addition of insulin-like growth factor 1 (IGF1) or activation of protein kinase B (PKB), causes muscle hypertrophy. In addition, inactivation of downstream components of mTOR causes muscle atrophy. Mice deficient for raptor or rictor in skeletal muscle are born in a Mendelian ratio. While rictor-deficient muscle can not be distinguished from wild-type muscle (Fig. 3A), raptor deficiency causes the muscle to become dystrophic as indicated by the presence of centralized myonuclei and occurrence of "central cores" (Fig. 3A). Moreover, these muscles have a higher content of glycogen but a lower capacity for oxidative phosphorylation. As a consequence, the raptor knockout mice have a significantly lower capacity to run long distances as revealed by voluntary wheel running (Fig. 3B). These experiments therefore show that mTORC1, but not mTORC2, is essential for the proper functioning of skeletal muscle. Furthermore our study reveals a novel role of mTORC1 in the regulation of the intrinsic metabolic properties of muscle fibers. Our results also suggest that a prolonged treatment of patients with rapamycin may cause pathological changes in the skeletal muscle.

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Specific dendritic signaling and its role neural network function

Almost all nerve cells in the central nervous system possess dendrites. The specific pattern of dendritic arborization serves as one of their characteristic features and is important in their classification. The vast majority of synaptic contacts between nerve cells involve a postsynaptic dendritic compartment and most of the surface of a neuron is usually represented by its dendrites.

Signaling in dendrites has always attracted a lot of attention in neurobiology; however, methods to directly investigate it have only become available within the last ten to fifteen years. As a consequence, the old prevailing notion of the dendritic tree as a passive recipient of synaptic input has only recently been revised, when it was found that signals are actively generated and can also actively propagate in dendrites.

One of the most useful technical advances was the development of high-resolution, high-speed imaging and the respective dyes, most notably calcium- and voltage-sensitive fluorophores. We are using these techniques together with molecular tools, which allow pharmacological targeting of specific $GABA_A$ receptors. It was recently discovered that distinct receptor subtypes fulfill different roles in regulating higher CNS functions and behavior. It was however unclear how these different functions were mediated at the network and cellular level. $GABA_A$ receptors are hetero-pentameric chloride channels, the most abundant configuration consists of two α two β and one γ subunit. Several isoforms (α1-6, β1-3, γ1-3) for each subunit can combine to form a diverse population of receptor subtypes. These subtypes differ in their affinities for GABA and modulatory ligands, in their kinetics and conductance and their subcellular targeting. The differences are, however, too subtle to easily distinguish the different receptors. The positive modulator diazepam binds to four subtypes of $GABA_A$ receptors, characterized by the presence of an α1, α2, α3, or α5 subunit together with a γ2 subunit with a single amino acid in the α subunit that determines diazepam binding (e.g. histidine at position 101 for the α1 subunit). Mutating these subunits in α1, α2 α3 α5 subunits created for the first time a selective pharmacological sensitivity and thus enabled dissociation of specific inhibitory effects.

Hippocampal $GABA_A$ receptors mediating phasic and tonic inhibitory signals

Morphological data indicated that different subtypes of $GABA_A$ receptors can be found at different types of synapses at hippocampal pyramidal cells and

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since these synapses are formed onto different parts of the neuron a characteristic pattern of receptor distribution is found on hippocampal pyramidal cells. We wanted to investigate the involvement of different $GABA_A$ receptor subtypes in different inhibitory signals in the hippocampus – using the point mutated mouse lines do determine the subunit composition pharmacologically. We found that signals that originate in proximal compartments of the cells were mainly mediated by α2 subunit containing receptors, whereas receptors mediating distal signals contained predominantly α1 subunits. We further determined that slow GABAergic signals mediated by spillover of the transmitter from the synaptic cleft were mediated by α5 and likely α1 subunit containing neurons. The special role of α5 subunit containing receptors is also underscored by their involvement in tonic inhibition. In summary: In the hippocampus different inhibitory signals are mediated by specific subsets of $GABA_A$ receptors that occupy specific subcellular locations (Prenosil *et al.* 2006).

Dendritic GABAergic signals in cortical pyramidal cells

It is thus clear that different subtypes of $GABA_A$ receptors act at specific sites on soma and dendrites of pyramidal cells. In order to study the impact of specific GABAergic receptors on dendritic signals we investigated the effect of diazepam on dendritic action potentials. In distal dendrites, calcium action potentials can be generated by coincident backpropagation of action potentials or by somatic burst firing. Above a given critical action potential frequency distal dendrites start firing calcium spikes.

Surprisingly, application of diazepam in wild type mice led to a significant reduction in the critical frequency. In α1 point mutated mice the reduction was no longer significant and in α2 subunit point mutated mice diazepam caused an increase in the critical frequency. In the somatosensory cortex α2 subunit containing receptors are found predominantly on the distal dendritic compartment (layers I-III). Excitatory effects mediated through $GABA_A$ receptors are commonly associated with increased intracellular chloride concentration. We therefore tested three different chloride concentrations (5, 15 and 30 mM) and found an excitatory effect only at the lowest concentration. The critical frequency is therefore mediated by a hyperpolarization of the dendrite. We were able to confirm this via direct ionophoretic application of GABA to either the soma or dendrites of pyramidal cells. In physiological chloride concentration,somatic application was inhibitory whereas dendritic application was excitatory. T-type calcium channels present the most compelling mechanism to translate a hyperpolarization into a reduced threshold for calcium action potentials. Indeed, blocking T-type calcium channels with nickel caused an increase in the critical frequency and a loss of the diazepam effect.

To summarize: Cortical pyramidal cells show a surprising degree of compartmentalization of the GABAergic system. Hyperpolarization at the soma decreases excitability on the one hand, on the other hand a novel type of excitatory hyperpolarizing GABAergic response is mediated by dendritic T-type calcium channels and α2 subunit containing $GABA_A$ receptors. These results will be published shortly.

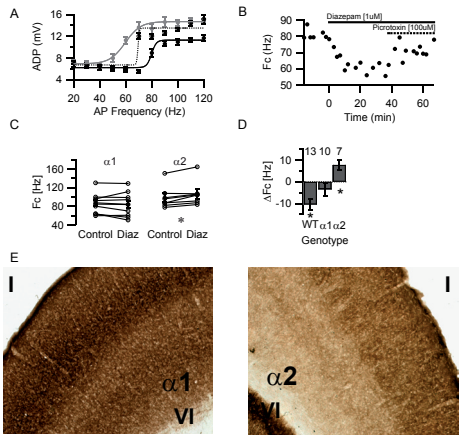

Figure 1: Diazepam paradoxically increases dendritic excitability.
A) *The frequency necessary to produce a dendritic calcium spike under control conditions (black solid) under diazepam (grey) and after picrotoxin (dotted). Note the sharp increase in the measured effect (Afterdepolarization (ADP) at the so-called critical frequency.* ***B)*** *The time course of the critical frequency (Fc); drugs are added at the indicated times. Diazepam reduces Fc and the GABAA receptor blocker picrotoxin reverses this effect. C&D) The effect of diazepam is reduced in mice carrying diazepam insensitive α1 GABAA receptor subunits and becomes inhibitory in mice carrying α2 GABAA receptor subunits. Thus, the excitatory effect of diazepam is mainly mediated by α2 subunit containing GABAA receptors.* ***C)*** *The individual experiments with Fc before and after diazepam. D) Summary of the effects in the mutant mice compared to the change in critical frequency (ΔFc) with wild type mice .* ***E)*** *Low power immunohistichemical images of the distribution of α1 and α2 subunits in mouse cortex from layer I to layer VI. Note the relatively homogeneous distribution of α1 subunits and the concentration of α2 subunits in layers I to III, where the distal apical dendrites of pyramidal cells are found.*

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Combined calcium and voltage imaging

Their elaborate geometry and their ability to actively generate signals allow dendritic trees to produce extremely complex electrical signals. In order to measure these signals with both high temporal and spatial resolution, we further developed and refined combined voltage and calcium imaging techniques. This requires the application of lipophilic voltage sensitive dyes to the inside of the neuron to be investigated via a patch electrode. However, since these dyes are toxic at higher concentrations the first patch pipette has to be withdrawn and the cell is then re-patched with a second electrode containing the calcium sensitive dye. We have successfully applied this technique to several cell types, but are focusing our efforts on cerebellar Purkinje cells at the moment, since these possess an essentially flat dendritic tree, which can thus be easily kept in focus. A paper detailing the methodological advances has been submitted (2007).

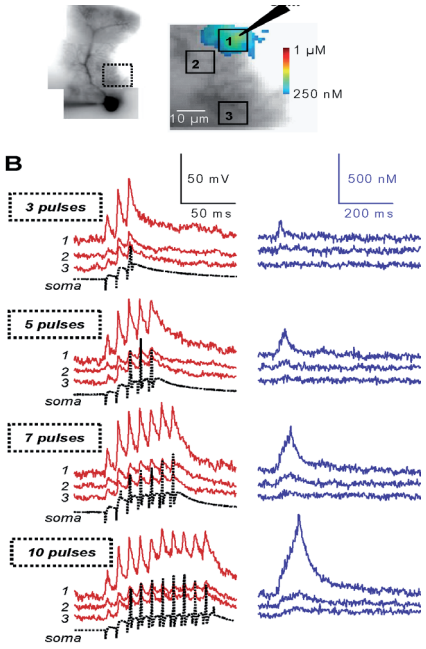

Figure 2: Double labeling with voltage sensitive and calcium dye in a cerebellar Purkinje cell.
A) Outline of the Purkinje cell in dark – on the right hand side the calcium signal elicited with the stimulus electrode (stim) is shown as a flase color overlay. Note the local nature of the calcium transient. B) Time course of somatic electrical recording (black), dendritic voltage signal (red) and dendritic calcium signal (blue) at increasing stimulus strength. Three different dendritic segments are shown (see above for location). Note the highly non-linear and localized calcium signal.

Cooperative plasticity in the cerebellum

Purkinje cells receive three different types of excitatory inputs, parallel and ascending fibers from cerebellar granule cells and climbing fibers from the inferior olive. The best known type of synaptic plasticity in the cerebellum is a long-term depression due to co-activation of parallel fiber and climbing fiber inputs. Recently long-term potentiation of parallel fiber inputs has been described, however, its exact mechanism and the role of cooperativity in this phenom-

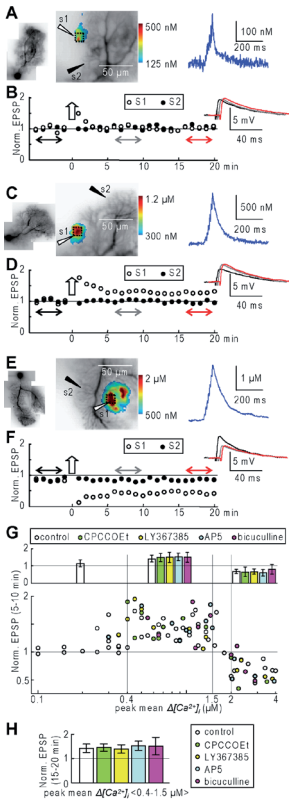

Figure 3: Calcium influx dependent synaptic plasticity of parallel fibers in Purkinje cells. Same method used as in figure 2. Two pathways (test: open circles and control: black dots) are used – only one undergoes the plasticity inducing treatment. A&B) Low calcium concentrations (blue trace) cause no change in the synaptic strength. C&D) Intermediate levels of calcium (blue trace) cause a persistent increase of the test pathway. E&F) High concentrations (blue trace) of calcium induce a depression of the test pathway. G) Summary of the effects. EPSP changes as a function of the postsynaptic peak calcium concentration. Note the limits at 0.4 and 1.5 µM increase in postsynaptic calcium. H) Pharmacological effects of various Blockers of metabotropic glutamate, NMDA receptors and a GABAB receptor antagonist. The effect is not mediated by receptors usually associated with synaptic plasticity, since the drugs have no effect.

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enon are unclear. With the help of combined voltage - and calcium imaging and pharmacology we were able to show that the crucial determinant of parallel fiber plasticity is local level of postsynaptic calcium. Low levels of calcium have no effect on synaptic efficacy, while intermediate levels lead to strengthening of synapses; high concentrations of calcium at the postsynaptic lead to a weakening of synapses at the site of high calcium. This is the inverse of the calcium-plasticity relationship observed in most other neurons in the brain. We are currently investigating the surce(s) for the calcium and the mechanisms translating the elevated calcium levels into altered synaptic strengths.

Stem-cell derived neural networks

Network formation in stem-cell derived neuronal cultures

By treating them with retinoic acid, stem-cells can be induced to differentiate into neurons. As these cells mature and acquire a neuronal phenotype they start forming synaptically connected networks of neurons. Both excitatory and inhibitory connections can be found, resulting in a functional equilibrium between excitation and inhibition. Blocking $GABA_A$ receptors drastically alters the balance and results in the occurrence of epileptic like discharges in these cultures. We are interested in the mechanisms that allow these cultures to achieve the observed equilibrium. We are currently investigating the effects of early manipulations of the inhibitory excitatory balance on cell differentiation and network homeostasis.

MeCP2 knockout cell lines as a cellular test system of Rett Syndrome

Rett-syndrome is the leading cause of inherited mental retardation in women. It is caused by dominant mutations in the X-chromosomal MeCP2 gene, which codes for an inhibitor of transcription that binds to methylated DNA. Recently, it was shown that reintroducing MeCP2 function in the CNS of MeCP2 knockout mice was able not only to halt progression of the disease, but to completely reverse the pathology. It is therefore not so much a developmental disturbance than an ongoing functional defect that underlies Rett syndrome pathology. We were using stem-cell derived neuronal cultures to assess the effect of MeCP2 deletion on neural differentiation and network function. Neurons from wild type and MeCP2 knockout stem-cells developed normally in terms of their intrinsic signaling capabilities, however, the MeCP2 knockout cultures showed a significantly altered amount of functional connectivity. We are currently verifying the results with additional cell lines.

In summary: Networks of neurons derived from MECP2 deficient stem-cells showed a different connectivity compared to wild type cultures. These results formed the basis of S. Cortesi's master thesis (2007) "Funktionelle Analyse von MeCP2-knockout-Neuronen als zelluläres Modell des Rett-Syndroms".

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Core Program Computational & Systems Biology and SIB – Swiss Institute of Bioinformatics

Computational & Systems Biology are interdisciplinary approaches, at the crossroads of life sciences, mathematics and information technology. They aim to build a global perspective of biological systems, from which one can infer novel organizational and functional principles. The combination of these approaches and large-scale, accurate experimental measurements are transforming biology into a modern, quantitative science.

The "Core Program Computational & Systems Biology" of the Biozentrum Basel consists of research groups headed by Mihaela Zavolan (RNA Regulatory Networks), Erik van Nimwegen (Genome Systems Biology), Torsten Schwede (Computational Structural Biology), Urs Meyer (Pharmacogenomics), and the associated group of Peter Philippsen (Applied Microbiology). Michael Primig (Microarray Data Management and Analysis) has left the University of Basel in 2007 to accept a position at the INSERM in Rennes.

Scientific Collaborations

The members of the Core Program Computational & Systems Biology are involved in a number of collaborative projects with both computational and experimental groups. This includes research groups within the Biozentrum, the University and other groups in the Basel area, collaborations on a Swiss-wide scope, e.g. within the framework of SIB or SystemsX.ch, and international collaborations with well known research organizations or networks such as the Riken Institute, the Research Collaboratory for Structural Bioinformatics hosting the Protein Data Bank, the European Bioinformatics Institute (EBI), the Rockefeller University, the Novartis Institute for Tropical Diseases in Singapore (NITD), and the European Alternative Splicing Network (EURASNET). For details, please refer to the individual research reports.

Teaching

The members of the Core Program Computational & Systems Biology are involved in a variety of teaching and training activities at the Biozentrum. They organize the graduate teaching program "Cycle F: Genomics and Bioinformatics", and have just introduced a B.Sc. teaching program in "Computational Biology" as part of the "Computational Sciences" curriculum. The Bioinformatics groups are participating in the Swiss-wide graduate school of the Swiss Institute of Bioinformatics, which has been awarded a Pro*Doc grant by the Swiss National Science Foundation. In

Professors
Urs A. Meyer, Michael Primig*, Torsten Schwede (Core Program Speaker), Erik van Nimwegen, Mihaela Zavolan

Associated Groups
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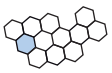


Core Program
Computational & Systems Biology
and SIB – Swiss Institute of Bioinformatics

collaboration with the Swiss EMBnet node, Lorenza Bordoli provides user support and practical training courses in Bioinformatics.

Infrastructure

In a collaborative effort between the Biozentrum, the Swiss Institute of Bioinformatics (SIB), and the Friedrich Miescher Institute (FMI), we are supporting competitive IT infrastructure that includes application-, database-, and web-servers, large scale storage and backup facilities for the Bioinformatics research groups, and various services for the entire Biozentrum. However, in 2006/2007 the necessary extension of the IT hardware infrastructure has been hampered by the limited capacity of server room space available at the University of Basel.

[BC]^2 Basel Computational Biology Conference

The Basel Computational Biology Conference [BC]^2 is an annual interdisciplinary symposium which is now established as the major Computational Biology event in Switzerland. It provides a platform for exchange of ideas among scientists from academic research institutes, the pharmaceutical industry and Biotech companies. The theme of the 4th [BC]^2 conference in March 2006 was “Comparative Genomics”, while the 5th [BC]^2 conference was organized in March 2007 together with the annual USGEB meeting to celebrate Leonhard Euler’s 300th birthday. Its motto was “From Euler to Computational Biology: Integrating mathematics into biological research”.

Pharmacogenomics

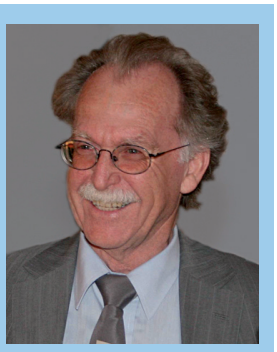
Genetic, environmental and host factors cause clinically important interindividual differences in the response to drugs and chemicals. Genetic polymorphisms of the genes coding for drug metabolizing enzymes, receptors and transporters are major contributors to this variability. A second major source of variability is the effect of drugs on gene expression, i.e. the transcriptional activation or repression of genes coding for drug-metabolizing enzymes, such as cytochromes P450, a phenomenon known as „induction“. The goal of our research is to understand the molecular mechanisms of variability in human drug response and to develop simple, non-invasive methods by which individuals at risk to develop adverse drug reactions, to suffer from drug inefficacy or drug-induced disease can be recognized.

Pharmacogenetics and Pharmacogenomics

Genetic polymorphisms of drug response are relatively common (>1%) monogenic traits caused by the presence of mutant alleles at the same gene locus and more than one phenotype in regard to drug interaction with the organism. Pharmacogenetic polymorphisms lead to subgroups in the population with altered responses to drugs and chemicals, e.g. a higher incidence of adverse drug reactions or a higher risk to develop a certain disease, for example cancer. In the past, we have elucidated the molecular mechanisms of several common genetic polymorphisms of drug metabolizing enzymes. For review, see Nature Rev./Genetics 5, 669-676, 2004. DNA tests derived from these projects are now being used in epidemiological studies throughout the world.

Mechanisms of transcriptional regulation of cytochrome P450 (CYP) genes by drugs

A second major source of variability in drug response is the effect of drugs on gene expression, i.e. the transcriptional activation or repression of genes coding for drug-metabolizing enzymes and drug transporters. The goal of our research team is to understand the molecular details of induction of drug-metabolizing enzymes and drug transporters. The following concept has emerged from studies in this and other laboratories. Inducers interact with the orphan nuclear receptors CAR (constitutive active or androstane receptor) or PXR (pregnane X receptor). These transcription factors form heterodimers with RXR (retinoid X receptor) and bind to enhancer motifs in the flanking regions of drug-regulated genes. We have identified enhancer motifs in several drug-induced genes notably cytochromes P450 CYP2H1,



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CYP3A37 and 5-aminolevulinic acid synthase (ALAS1), the first and rate-limiting enzyme in heme biosynthesis. Moreover we have discovered a new xenobiotic-sensing nuclear receptor, named CXR (chicken xenobiotic-sensing receptor) and have demonstrated important links between cholesterol and bile acid homeostasis and drug metabolism. We also have developed a non-mammalian system, namely avian liver, avian hepatocytes and avian hepatoma cells (LMH), to study induction of cytochromes P450 and heme synthesis by drugs and chemicals. In LMH cells cytochromes P450 remain highly inducible by phenobarbital-like inducers in contrast to mammalian cell lines. One of our goals is to develop cell lines derived from human hepatoma cells that maintain the response to phenobarbital. Our experiments in LMH cells provide strong evidence for evolutionary conservation of the signaling pathways triggered by PXR, CAR and CXR. We could demonstrate that closely related nuclear receptors, transcription factors and signaling pathways are mediating the transcriptional activation of multiple genes by xenobiotics in chicken, rodents and man (Handschin *et al*., 2002, 2005; Meyer, 2007). However, the reasons for the lack of drug induction in mammalian cell-lines remains obscure.

How do drugs activate PXR and CAR?

There are major open questions regarding the mechanism by which drugs activate gene transcription. PXR and CAR both are mediators of the cellular response to xenobiotics, but their mechanisms of activation are different (Handschin & Meyer, 2003). PXR is directly activated by xenobiotics that bind to its ligand-binding domain. CAR is unusual among the nuclear receptors in that it has high constitutive activity and is predominantly located in the cytoplasm. Treatment with phenobarbital, the classical inducer of drug metabolism, results in the translocation into the nucleus, where CAR (as heterodimer with RXR, the retinoid X receptor) transactivates the phenobarbital-response elements (PBRUs) or PBREMs of P450s and other genes. The mechanisms by which phenobarbital-type inducers lead to the translocation of CAR are not known, nor are those by which CAR transactivates the PBRUs. Franck Rencurel in our team has discovered that the energy sensor AMPK (AMP activated kinase) is necessary for phenobarbital-type induction in a human hepatoma-derived cell line (Rencurel et al., 2005). He now has reproduced and extended these findings in human hepatocytes in primary culture (Rencurel *et al*., 2006). Sharon Blättler has studied induction in LMH cells, providing evidence for an evolutionary conservation of this mechanism. In both human and chicken hepatocytes, phenobarbital increases the activity of AMPK. Within her PhD thesis, Sharon Blättler discovered that the mechanism of AMPK activation is related to the effect of phenobarbital-type inducers on mitochondrial function with consequent formation of reactive oxygen species (ROS) and phosphorylation of AMPK by its upstream kinase LKB1. With gain- and loss of function experiments, Sharon Blättler could demonstrate that LKB1-activated AMPK is necessary in the mechanism of drug induction both in LMH cells (Blättler *et al*., 2007) and in human hepatocytes in primary culture. The protein targets, however, are still unknown.

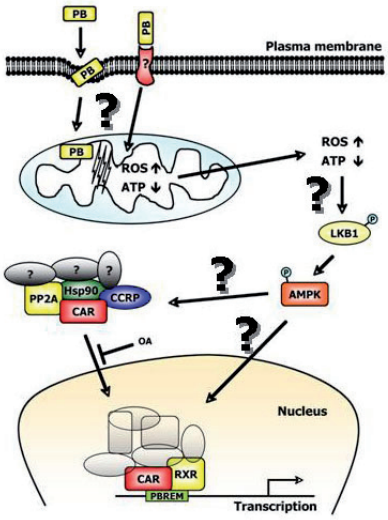

Figure 1: Working model for the mechanism of Phenobarbital (PB) activation of transcription of drug-metabolizing enzymes.

Crosstalk between drug metabolism and energy sensing in hepatocytes

Phenobarbital not only induces drug metabolism but has multiple other effects. The observation that phenobarbital-type inducers lower glycemia in patients with diabetes by repressing genes involved in gluconeogenesis, e.g. phosphoenopyruvate carboxykinase (PEPCK) suggested an effect on energy metabolism. Within his PhD thesis, Taoufiq Harach could demonstrate that activation of AMPK is necessary but not sufficient to trigger the effect of phenobarbital on PEPCKmRNA. His experiments in mice with liver-specific deficiencies of AMPKα1 and α2 subunits and in mice with targeted deletions of CAR now suggest that phenobarbital, by activating AMPK, represses the interaction between CAR and PGC1α,

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the peroxisome proliferator activated receptor 1α, a known regulator of PEPCK. This now provides a new mechanism for the interaction of inducing drugs with energy metabolism (Harach *et al*., submitted).

Crosstalk between xenobiotic-sensing nuclear receptors and receptors involved in cholesterol and bile acid homeostasis

We have continued our studies on the crosstalk between the nuclear receptors involved in cholesterol and bile acid homeostasis, namely LXR (the oxysterol receptor) and FXR (the bile acid receptor) and the xenosensing nuclear receptors PXR, CAR and CXR. By feeding mice a 1 % cholesterol diet, Adrian Roth discovered that a target gene of LXR, Srebp1, directly interferes with coactivator recruitment by PXR and CAR and can explain the repression of xenobiotic metabolism by cholesterol (Roth *et al*., in revision). Adrian Roth also discovered that activators of CAR and PXR induce Insig1, a protein with antilipogenic properties, explaining the effect of inducers on triglycerides. These data also explain clinical observations in rodents with disturbed lipid metabolism (e.g. the Fa/Fa Zucker rat) and in obesity in man.

At another level of regulation, Diana Jung has established that the bile acid receptor FXR regulates PXR expression by several FXR recognition sites in the promoter of PXR. This represents an additional mechanism for the elimination of high levels of bile acids (Jung *et al*., 2006). It may be relevant for the clinically important extremely variable expression of CYP3A4. Taken together, these recent experiments reveal an interesting coevolution of detoxication mechanism in the liver for drugs and bile acids.

Within the framework VI European project „Steroltalk“ we have contributed to the development of a cDNA microarray which enables systemic studies of transcription of 300 genes involved in cholesterol homeostasis and drug metabolism in mouse and human liver (Rezen *et al*., 2007). Viola Tamasi has used these microarrays to study the relative contribution of CAR and PXR in the regulation of cytochromes P450. Mice with targeted deficiencies of CAR, PXR and socalled „double knockouts“ for CAR and PXR were treated with inducers of CAR (TCPOBOP) and PXR (PCN) and with phenobarbital. As an example, in the absence of CAR and PXR, phenobarbital induced the mRNAs of other nuclear receptors, including PPARα and did not induce AMPK. The mechanism of this unexpected crosstalk between different nuclear receptors is presently investigated.

Role of heme synthesis during induction of cytochromes P450

Induction of cytochrome P450 (hemeproteins) requires a coordinated increase in heme synthesis. In previous studies, we identified several drug-responsive enhancer elements in the flanking region of the chicken, mouse and human 5-aminolevulinate synthase gene (ALAS1), the rate-limiting enzyme of heme synthesis. The chicken, mouse and human enhancers have been published (Fraser *et al*., 2002; 2003, Podvinec *et al*., 2004). In an extension of these investigations, we have studied the effect of fasting on the regulation of ALAS1 in collaboration with Christoph Handschin. We have discovered that ALAS1 is regulated by PGC-1α (Handschin *et al*., 2005). These findings allowed us to restudy the mechanism of increase in heme synthesis during the induction of cytochromes P450 and to search for additional elements that respond to other inducer classes, a project carried out by Anne-Kathrin Peyer. By analyzing ultraconserved DNA sequences in the 5‘flanking region of chicken, mouse and human ALAS1, a number of recognition sites for the transcription factor Hepatic Nuclear Factor 4α (HNF4α) and for FXR were discovered. These sites have been studied by mutagenesis, chromatin immunoprecipitation in primary human hepatocytes and human liver slices. These data strongly support a role of bile acid activated FXR in the regulation of human ALAS1 and consequently hepatic porphyrin and heme synthesis. The data also suggest that bile acids could precipitate acute attacks of hepatic porphyrias which are associated with increased ALAS1 activity (Peyer *et al*., 2007).

In summary, all our experiments are designed to ultimately evaluate genotype-phenotype relationships in drug response and develop predictive tests for personalized medicine.

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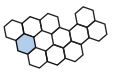

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Control of meiosis and gametogenesis in yeast and mammals

Progression through meiotic development is in part controlled by an expression program that coordinates timing of induction and time of function of numerous loci essential for the process. To study this program and the regulatory network that drives it, our lab is carrying out genome-wide transcriptional profiling experiments of meiosis and gametogenesis in budding yeast and male mammals. In parallel, we develop array data management and analysis solutions.

The yeast meiotic transcriptome

Diploid budding yeast cells lacking nitrogen and a fermentable carbon source undergo meiotic development that leads to the formation of a four-spored ascus. We have studied this process in a well characterized fast sporulating strain (SK1). A time-course experiment was carried out in duplicate including heterogenous cell populations growing in rich medium supplemented with glucose (YPD) or acetate (YPA) and cells incubated in sporulation medium (SPIII) until 10 hours after induction of meiosis. Target synthesis was done in collaboration with L. Steinmetz (EMBL, Heidelberg) and yeast tiling arrays were provided by RW Davis (Stanford University, Palo Alto). Preliminary raw data analysis reveals excellent data quality and reproducibility. We are now in the process of mapping transcripts back onto the genome sequence to confirm annotated open reading frames and to discover new protein-coding and non-coding loci. Further work will include functional characterization of novel meiotic protein-coding and non-coding transcripts (A. Lardenois, T. Walther, F. Chalmel, P. Demougin, L. Steinmetz, R. Davis and M. Primig, unpublished)

UME6: Sporulation depends upon mitotic repressors of meiotic genes (Ume6 and Sum1), a general regulator (Abf1) and a meiosis-specific activator of middle and mid-late genes (Ndt80) that bind regulatory motifs present in meiotic promoters. The onset of meiosis is concomitant to the destruction of Ume6 repressor by the APC/C protease in a process that is dependent upon Ime1. To better understand the role of Ume6 during mitotic repression and why cells that lack the repressor fail to sporulate, we carried out expression profiling studies using wild-type and mutant strains. By combining array data with Ume6 motif prediction and genome-wide DNA binding assays we discovered 123 putative direct target genes including all loci previously known to be regulated by Ume6 (T. Walther, Y. Liu, I. Erb, P. Demougin, E. v Nimwegen and M. Primig, in preparation).

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ABF1: The DNA-binding Autonomously Replicating Sequence Binding Factor 1 (Abf1) was initially identified as an essential DNA replication enzyme and later shown to be a component of the regulatory network controlling mitotic and meiotic cell cycle progression in budding yeast. The protein is thought to exert its functions via specific interaction with its target site as part of distinct protein complexes but its roles during mitotic growth and meiotic development are only partially understood. We carried out a comprehensive approach aiming at the identification of direct Abf1-target genes expressed during fermentation, respiration and sporulation. Computational prediction of the protein's target sites was integrated with a genome-wide DNA binding assay in growing and sporulating cells. The resulting data were combined with the output of expression profiling studies using wild-type versus temperature sensitive alleles and protein-protein interaction data. This work identified 434 protein-coding loci as being transcriptionally dependent on Abf1. More than 60% of their putative promoter regions contained a computationally predicted Abf1 binding site and/or were bound by Abf1 *in vivo*, identifying them as direct targets. Our work yielded numerous loci previously unknown to be under Abf1 control and revealed the protein's unexpectedly variable DNA binding pattern during mitosis and meiotic development. Finally, we found a role for Abf1 in coordinating cytokinesis and spore wall formation via UAS-mediated transcriptional control of genes encoding septin ring components (Schlecht *et al*., submitted).

The core meiotic transcriptome in mammals

Whole-genome profiling of male germline expression in mouse, rat and human provides important clues about the transcriptional regulatory machinery that drives the expression of its target genes. A subset of conserved loci expressed in testicular tissue show highly similar meiotic and post-meiotic profiles across mouse, rat and human. Mouse genes specifically transcribed in the germline are often important for sexual reproduction. Computerized promoter analysis reveals the degree of intergenic DNA sequence conservation and the localization of known regulatory elements within putative promoter regions tha control meiotic and post-meiotic genes. Some genes strongly expressed in testis are implicated in somtic malignancies suggesting that gametogenesis and tumorigenesis share genetic traits. Our study revealed the kinetics of conserved and co-expressed germline genes across species and will facilitate the identification of novel genes important for male gametogenesis and fertility (Spiess *et al*, Hum Reprod. 2007; Chalmel *et al*., PNAS 2007; Chalmel *et al*. Ann NY Acad. Sci. 2007).

Microarray data management and analysis

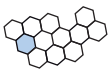
The high-density oligonucleotide microarray (GeneChip) is a popular tool for large-scale detection of small nucleotide polymorphisms in DNA and changes in mRNA concentrations. Array data management solutions are crucial for data analysis and interpretation. To organize high-throughput expression profiling experiments, the Microarray Information Management and Annotation System (MIMAS) was developed and installed. It is compliant with the Minimal Information About a Microarray Experiment (MIAME) convention. and provides researchers with a flexible GeneChip data storage and annotation platform. The MIMAS system software is freely available for academic users. The database is accessible for registered users at http://.mimas.vital-IT.ch/ (Hermida *et al*., BMC Bioinformatics 2006).

The Ashbya Genome Database

The Ashbya Genome Database (AGD) 3.0 is a cross-species genome and transcriptome browser that provides information on 4726 protein-encoding loci and 293 non-coding RNA genes present in the genome of the filamentous fungus Ashbya gossypii. A synteny viewer shows the chromosomal location and orientation of orthologous genes in budding yeast. Whole-genome expression profiling data obtained with high-density oligonucleotide microarrays (GeneChips) have been integrated for most of the currently annotated protein-coding loci in A. gossypii and budding yeast. AGD 3.0 provides users with report pages including DNA annotation from Ensembl, Gene Ontology terms associated with S. cerevisiae orthologues and array profiling data as well as useful external links. The database is accessible at http://agd.vital-it.ch/ (Gattiker *et al*., BMC Genomics 2007).

The GermOnline Systems Browser

We have developed and put online a novel release of the GermOnline knowledgebase focussing on data relevant for the cell cycle, gametogenesis and fertility across popular model systems and human. The database was extended into a systems browser providing information on DNA sequence annotation (Ensembl), gene expression (GeneChips) and the function of gene products (GeneOntology). The database covers the genomes and transcriptomes of eight model organisms and Homo sapiens. GermOn-



line is built around a sophisticated genome browser (Ensembl), our own microarray information management and annotation system (MIMAS) and a comprehensive system for online editing of database entries (MediaWiki) that was originally developed for the popular online Wikipedia project. Expression data include classical and tiling arrays; the latter yield information on transcriptional levels, mRNA start and termination sites and exon composition (alternative splicing). The database is accessible at http://www.germonline.org/ (Gattiker *et al.*, NAR 2007).

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Computational Structural Biology

One of the major bottlenecks of structure-based approaches in biomedical research is the limited availability of experimentally determined protein structures. Theoretical methods for protein structure prediction and molecular modeling are aiming to bridge this structure knowledge gap. The main goal of my group is to develop methods and algorithms to model, simulate and analyze three-dimensional protein structures and their molecular properties in order to apply these techniques to understand biological processes at a molecular level. Many of our projects include collaborations with experimental groups.

CASP7 - Critical Assessment of Techniques for Protein Structure Prediction Round VII

Protein structure modeling and prediction has matured and today is widely used as a scientific research tool. Consequently, it is ever more important to evaluate to what extent current prediction methods meet the accuracy requirements of different scientific applications. A good way to assess the reliability of different protein structure modeling methods is to evaluate the results of blind predictions *a posteriori* when the corresponding protein structures have been determined experimentally, e.g. during the biannual 'Community Wide Experiment on the 'Critical Assessment of Techniques for Protein Structure Prediction' (CASP). For a CASP experiment, participating research groups apply their prediction methods to a defined set of protein sequences, for which the experimental structure is about to be determined. The accuracy of these blind predictions is then evaluated independently once the structures are made available. We have been invited to assess the prediction results of the 7th round of CASP in the categories of "Disorder Prediction" and "Template-Based Modeling".

Besides using classical measures for assessing the accuracy of the C_α positions of the models, several additional criteria were introduced in CASP7 to ensure that the assessment appraises the overall quality of the models, as well as those features of the predictions that are relevant to their usefulness in specific scientific applications, such as the fraction of correctly modeled hydrogen bond interactions (HBscore), the suitability of models for phasing X-ray diffraction data, assessment of the accuracy of predicted cofactor binding sites, and accuracy of the model error estimates provided by the predictors. In this latest edition of CASP, the general trends observed in previous years continued: Comparative modeling remained by far the most accurate technique for protein structure modeling. However, the

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majority of predictions submitted in the category of template-based modeling (TBM) were again closer to the template than to the real structure, and only in few cases some improvement over a model based on a single best template structure was observed. The fact that no group would outperform a virtual predictor submitting models based on the single best template for each target indicates that template identification and alignment are by no means solved problems and constitute a major bottleneck, besides the challenging question of model refinement. However, successful refinement of model coordinates closer to the experimental structure has been observed at least in a number of cases (e.g. target T0283 model submitted by the Baker group).

One of the most remarkable results of CASP7 was that automated prediction servers have matured significantly in recent years: Six of the top twenty-five groups in the assessment of template based models were predictors using automated prediction servers, which produce their models without manual intervention. In 29% of a total of 108 cases, the best model for an individual prediction target was submitted by a server. The best prediction server (Zhang Server) was ranked third over all, i.e. it outperformed all but two of the participating groups. The results of the CASP7 assessment have been published in a special edition of the journal “Proteins: Structure, Function, and Bioinformatics”.

Expert systems for comparative protein structure modeling

Template-based protein modeling techniques (aka “homology modeling” or “comparative modeling”) exploit the evolutionary relationship between a target protein and templates with known experimental structures, based on the observation that evolutionarily related sequences generally have similar 3D structures. Currently, comparative protein structure modeling is the only computational approach, which can routinely generate three-dimensional protein structure models suitable for applications which require highly accurate structural information. Since nearly 15 years, the SWISS-MODEL server has pioneered automated web-based comparative modeling. My group is developing and operating the SWISS-MODEL server as part of our activities within the Swiss Institute of Bioinformatics. Over the last 12 months, our server has treated more than half a million requests for individual 3-dimensional protein models. We have established the SWISS-MODEL Repository, a relational database of annotated protein models, which has been well accepted as a resource for model information. SWISS-MODEL Repository is explicitly cross-referenced in the SwissProt and InterPro databases.

In an effort to provide an integrated access to the various worldwide databases containing structural information, we have developed the Protein Model Portal as part of the PSI Structural Genomics Knowledge Base in collaboration with RCSB PDB, thereby implementing the first step of the community workshop recommendation on archiving structural models of biological macromolecules (Berman *et al*., 2006). Currently, models from six structural genomics centers, MODBASE and SWISS-MODEL Repository are accessible through a single search interface.

The Dengue Docking Project

Dengue fever is a viral infection (genus Flavivirus) causing fever, severe joint pain, less often hemorrhage and shock and in the most severe cases, death. It is found in tropical and sub-tropical regions and over the past few decades the virus has spread dramatically, with 2 billion people living in areas with infection risk. At present, there is no known cure or vaccine for this disease. Dengue is among a group of ailments referred to as neglected tropical diseases (NTDs). For these diseases, populations most affected are also the poorest and most vulnerable and are found mainly in tropical and subtropical areas of the world. Diseases which affect mainly the developing countries are of limited commercial potential. Innovative public-private partnerships between non-profit

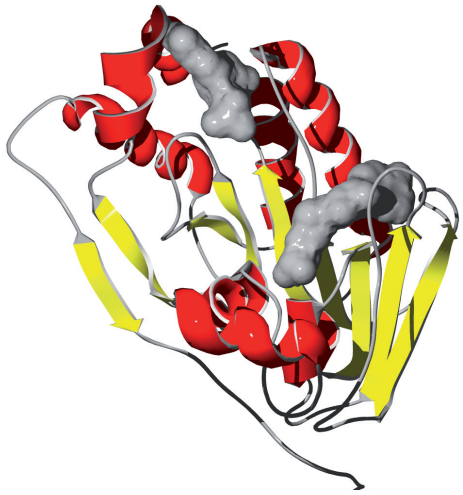

Figure 1: Structure of Dengue Virus NS5 Methyltransferase as target for large scale virtual screening for potential inhibitors. Binding sites of S-Adenosyl Homocysteine and Ribavirin 5‘ triphosphate are highlighted (PDB: 1r6a).

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organizations, such as universities or foundations, and industrial partners can give new momentum to the development of new drugs for neglected tropical diseases. We have established collaboration with the Novartis Institute for Tropical Diseases (NITD, Singapore) and Schrödinger Llc. in a joint effort to search for new antiviral agents against Dengue.

When structural data for a protein target are available, computationally 'docking' molecules into the protein structure and scoring their calculated binding affinities can be a valuable aid in selecting compounds which are likely to be active in screening assays. As a first Dengue virus target, we selected the methyltransferase function of the NS5 protein. Insights gained from the three-dimensional structure of the binding site allowed us to perform virtual screening of large libraries of commercially purchasable compounds to identify likely inhibitors. A key problem in docking is that assessing the interaction of all possible conformations, orientations and positions of a given ligand with even one rigid model of the protein receptor is expensive in terms of computational time, and so becomes impractical for screening huge libraries. Using grid computing, sufficient free compute capacity can be harvested from clusters and individual desktops to allow more extensive testing of larger sets of candidate molecules.

We have successfully screened over 6 million commercially available compounds using the GLIDE software package. Further post-processing for plausibility and chemical diversity resulted in a short list of promising candidate compounds. These compounds arising from the virtual screening are currently analyzed in vitro by the Novartis Institute for Tropical Diseases (NITD, Singapore). Ultimately, it is also the results from biochemical assays that allow us to validate and improve on the performance of the applied virtual screening strategy.

GRID computing as a platform for applied research in bioinformatics

Computational biology is often limited by the available computational resources. However, the combined theoretical computation power of Swiss scientific institutions, including unused desktop PCs, is orders of magnitude larger than that available at each individual institution. We have developed a computational GRID infrastructure for bioinformatics applications which allows us to transparently compute ‚embarrassingly' parallel calculations in a heterogeneous hardware and OS environment. The aforementioned Dengue docking project was the main motivation for this work. Additionally, we have grid-enabled the open-source peptide identification algorithm X!Tandem. An application service was developed that allows the parallel execution of peptide searches on a desktop PC grid consisting of hundreds of PCs. Users interact with this service through a web-based environment adapted from our SWISS-MODEL Workspace for protein homology modeling.

Structural and functional basis of odorant receptor mediated signaling

Olfaction, the detection of odorous compounds, is among the oldest of the sensory systems. It is ideally suited to study the complex mechanism of transducing chemical into neuronal signals generating behavioral responses, which are essential for the survival of most mammals. The interaction of volatile molecules with distinct G protein coupled olfactory receptors (OR), about 350 in human, is the central molecular event of detecting and discriminating thousands of odorants. As part of a SystemsX interdisciplinary pilot project, we have entered collaboration with the Vogel group (EPFL) in an integrated experimental and bioinformatics approach to elucidate the molecular basis of olfaction and its decoding into cellular responses: Functional screening of chemical libraries is used to develop computational models of the molecular specificity of distinct ORs which are not yet characterized for their ligand specificity. Iterative cycles of site-directed mutagenesis of receptor ligand binding regions combined with remodeling will refine the accuracy of the predicted

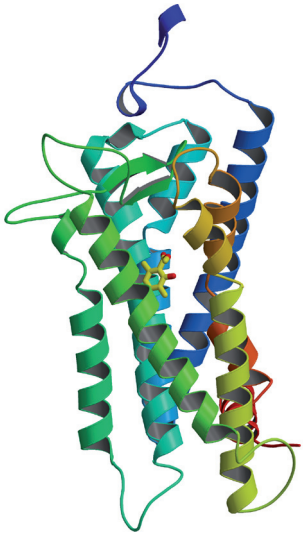
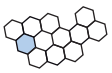


Figure 2: Model of the mouse Eugenol olfactory receptor in complex with odorant molecule.



receptor structures and specificities. We have first experimental and modeling results pointing to dual functions of some odorant compounds activating both ORs and endocrine receptors. Our discovery that a particular ligand is capable of activating two different receptors, involved in completely unrelated cellular functions, opens novel routes for analyzing basic principles of molecular recognition of chemical signals in biological systems.

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Principles of regulatory design

Introduction

The proper functioning of a cell crucially depends on a network of complex regulatory systems that control when different proteins will be expressed, when and where they will be activated, what molecules are transported in and out of the cell, and so on. These regulatory systems have been designed by an evolutionary process that has taken billions of years and we currently understand very little about the way in which this evolutionary process has shaped the design of these regulatory systems. The main research interest of our group is the study of genome-wide regulatory systems, to reconstruct them from high-throughput molecular data, to understand and model how they have evolved, and to search for design principles in their construction.

The activities in our group revolve around the development and application of computational and analytical tools to study regulatory networks and their evolution. These studies can be roughly separated into three components. First, we are developing new algorithmic tools for the automated reconstruction of genome-wide regulatory networks from comparative genomic, ChIP-on-chip, and gene expression data. Second, we are developing methods for studying genome evolution and the evolution of regulatory networks in particular. Finally, we are studying the properties of the inferred regulatory networks and build evolutionary models that can account for the observed characteristics.

Toward a grammar of yeast‘s transcriptional regulatory code

Over the last years a combination of high-throughput experimental data and newly developed computational methods have made it possible to identify the locations of transcription factor binding sites genome-wide for some model organisms. In particular for *S. cerevisiae* a draft of the genome-wide `transcriptional regulatory code‘ has been made by several groups including ours. Our most recent genome-wide annotations of regulatory sites in S. cerevisiae have been made available through the SwissRegulon database (www.swissregulon.unibas.ch) which also contains genome-wide annotations of regulatory sites in a number of bacterial genomes. In the last two years we have made an in-depth study of the `grammar‘ of this regulatory code. In particular we focused on studying the locations of transcription factor binding sites for different transcription factors both relative to each other and relative to transcription start sites genome-wide. First of all we uncovered that there is

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a substantial number of pairs of TFs that have significantly overlapping sets of target genes and that can be divided into roughly three categories: 1 “competing” TFs that bind to overlapping binding sites and therefore compete for binding to the same site. These TFs often (but not always) have highly similar DNA binding domains. 2 “complex-forming” TFs that prefer to bind at fixed relative distances and orientations and that presumably can form a complex when bound to the DNA simultaniously.3 “cooperating” TFs that act independently but that regulate genes in the same pathways. A second important grammatical feature that we found is that binding sites for every TF have particular positional preferences relative to the transcription start site. Moreover, these positional preferences reflect the mechanisms of function of different TFs. For example, there is a set of transcription factors (including ABF1 and REB1) whose sites occur preferentially around 110-120bps upstream of transcription start and that act by inducing a local nucleosome-free area. Third, as in higher eukaryotes, only about 1/5 of yeast promoters contain a TATA-box. Several lines of evidence suggest that a number of other TFs (including GATA and Forkhead factors) can substitute for TATA-binding protein at a significant number of yeast promoters. In particular, these TFs have roughly the same positional preferences as TATA-binding protein and they occur almost exclusively in promoters that do not contain a TATA-box. Finally, our analysis of the sequence composition in yeast promoters supports a model of transcription initiation in which the pre-initiation complex is recruted to the DNA somewhere between 60 and 100 bps upstream of transcription start, scans downstream from this position, and selects a transcription start site in a sequence-dependent manner.

Predicting protein-protein interactions from amino acid sequences: Inferring bacterial signaling networks

A method that comprehensively and accurately predicts protein-protein interactions using only the amino acid sequences of proteins would essentially allow the reconstruction of genome-wide protein interaction networks directly from genome sequences. Automated prediction of protein-protein interactions from their amino acid sequences is therefore one of the great outstanding challenges in computational biology. Over the last two years we have developed a novel Bayesian network model that predicts protein-protein interactions directly from amino acid sequences, without tunable parameters and without the need for any training examples. The method operates on sets of protein families for which it is known that members from one family interact with members from one or more of the other families. Multiple alignments of the sequences in each family are constructed and the algorithm searches over all possible ways in which the proteins from different families can be paired up to form interacting pairs. The best assignment of interacting pairs is roughly speaking the one that maximizes the statistical dependencies that are observed between amino acids of the interacting protein pairs (see figure 1).

We have applied the method to comprehensively reconstruct two-component signaling networks across all sequenced bacteria involving thousands of kinase and regulator proteins. Comparisons of the predictions with known interactions show that the method infers interaction partners genome-wide with high accuracy. Analysis of the predicted genome-wide two-component signaling networks reveals several interesting features. First, we find that cognate ki-

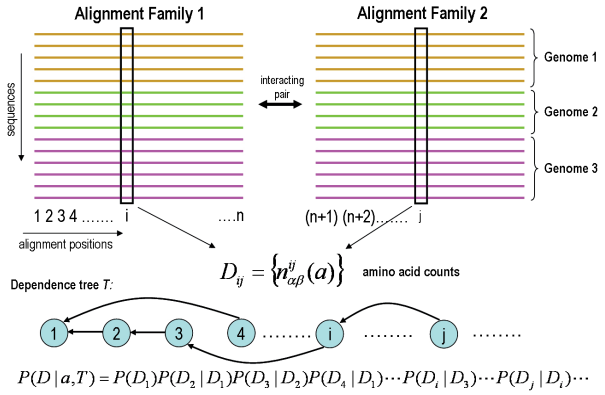

Fig 1:Illustration of the algorithm for protein-protein interaction predictions. The alignments of two interacting protein families are shown side by side, with sequences grouped per genome from top to bottom. In this simple example it is assumed each protein from the left family interacts with one member from the right family. An assignment of interaction partners a corresponds to a vertical ordering of the sequences within each genome such that the sequences on the same horizontal `row‘ are assumed to interact. In this way an assignment a implies a joint multiple alignment of all sequences of both families. The probability of this joint alignment is calculated using a probabilistic model that allows each amino acid residue to depend on exactly one other amino acid residue, either within the same protein family or in the other protein family. The resulting dependence structure is a tree and our algorithm determines the probability of an assignment a by summing over all possible dependence tree structures.

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nase/regulator pairs (which lie adjacent on the genome) and orphan kinases and regulators (which lie isolated) form relatively separate components of the signaling networks, i.e. orphans interact mostly with orphans and cognates mostly with cognates. Second, we find that the average number of interaction partners of each gene grows slowly with genome size. Finally, whereas most kinases and regulators interact with only a few partners, about 10% interacts with a large number of partners. Most of these `hub' kinases and regulators are orphans. The kinases in this class thus distribute a signal to a large number of downstream regulators, and the regulators in this class integrate a large number of input signals.

The structure of bacterial regulatory networks

With the aim of characterizing the global structure of transcription regulatory networks across bacteria of different size, we have performed an extensive comparative genomic study, involving 22 clades of related bacterial genomes. Using whole genome sequences of groups of related bacteria and newly developed computational methods we have quantified the patterns of selection at non-coding positions genome-wide across the different clades of bacteria. This analysis uncovered a number of striking features that are shared by all bacteria, and that give important insights into the organization of bacterial regulatory networks. For example, we found that in all bacteria there is significant evidence of selection at regulatory sites immediately upstream of gene starts. The strength and positional distribution of these selection patterns are essentially identical across clades and imply that the number of regulatory sites per gene is the same in all bacteria. This is particularly striking because our group has previously established that the total number of transcription factors increases quadratically with genome size. Together the two observations imply that in small bacterial genomes there is a relatively small number of TFs that each have a large number of regulatory targets, whereas in large bacteria there are many TFs that each have a small number of regulatory targets (see figure 2). This is supported by the fact that we observe that in small genomes the most conserved motifs in intergenic regions are also the most abundant whereas in large genomes the most conserved motifs are rare. We believe these results point to a fundamental difference in the regulation of prokaryotes and eukaryotes. In particular, it seems that in contrast to eukaryotes, prokaryotes have never developed the molecular mechanisms that allow a gene to be regulated by many different TFs and it is tempting to speculate that this may be one of the reasons why complex multi-cellular organizations are only observed in eukaryotes.

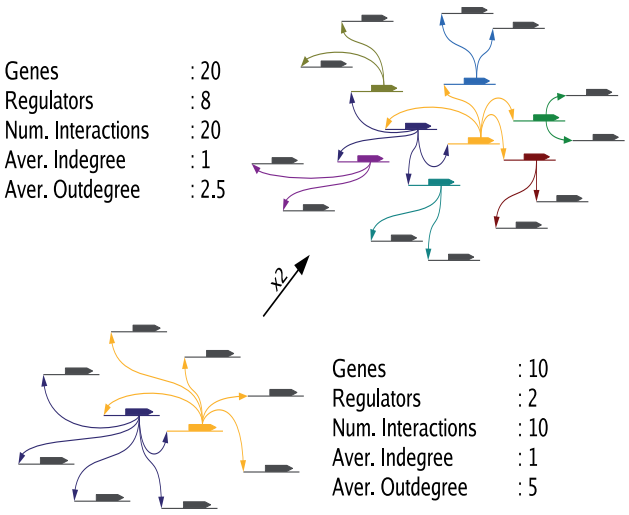

Fig 2: Schematic illustration of the structure of transcriptional regulatory networks across bacteria of different genome size. As the genome size goes from 10 to 20 genes, the number of transcription factors quadruples from 2 to 8. However, the total number of regulatory inputs per gene stays the same, i.e. 1., leading every transcription factor in the larger genome to have less regulatory outputs.

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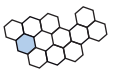

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Identification of regulatory RNAs and of their mRNA targets

Small RNAs have emerged as important regulators of gene expression in species ranging from bacteria to man. In mammals, the two most abundant classes of small regulatory RNAs are the microRNAs (miRNAs) and the Piwi protein interacting RNAs (piRNAs).

Analysis of miRNA expression

MiRNAs are produced from imperfect hairpin precursors by RNase III type enzymes Drosha and Dicer. The mature miRNA products are incorporated into an RNA-induced silencing complex (RISC), which also contains proteins of the Argonaute family. This complex, in which the miRNA acts as guide, targets mRNAs for translational silencing and at least partial degradation. Over the past few years, our group worked on the identification and expression profiling of miRNAs. We analyzed more than 330,000 independent small RNA sequences from 256 small RNA libraries that were prepared from 26 distinct organ systems and cell types of human and rodents [1]. In this process, we identified 33 new miRNAs, and we confirmed the expression of many evolutionarily conserved miRNAs previously only cloned in other species. Importantly, although the highest estimate of the number of human miRNAs has now reached tens of thousands, 97% of the sequences in our data set originated from less than 300 precursors that we called “prototypical”. These precursors have a precise pattern of processing, leading to a sharply defined 5’ end of the mature miRNA, are generally not repetitive, and show some evidence of cross-species conservation. Most of these miRNAs are expressed broadly and robustly, although their concentration is modulated from tissue to tissue. In a particular system that we studied, namely the hematopoietic system, comparisons of the expression profiles of various cell types indicate that only few miRNAs have cell type-specific expression, whereas most of the differences between these cells consist in relative changes in the expression of broadly-expressed miRNAs. One of the outcomes of our study was a miRNA expression atlas (www.mirz.unibas.ch/smiRNAdb) that documents the miRNA profiles of the tissues and organs that we studied, and that is already used to support future research on miRNA function and its implication in disease.

Prediction and experimental identification of miRNA targets

With hundreds of human miRNAs deposited in sequence databases, the focus has now shifted towards the characterization of their targets. In order to guide the experiments, several computational tools to pre-

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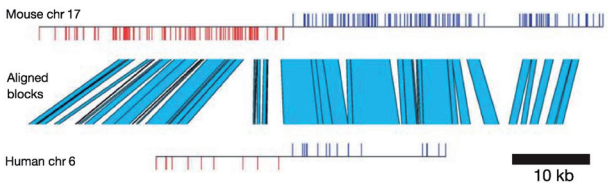
dict miRNA targets have been developed. Our group developed a general Bayesian method [2] in which, for each miRNA, we explicitly model the evolution of orthologous target sites in a set of related species. Using this method we predicted target sites for all known miRNAs in flies, worms, fish, and mammals. By comparing our predictions in fly with a reference set of experimentally tested miRNA-mRNA interactions we showed that our general method performs at least as well as the most accurate methods available to date, including ones specifically tailored for target prediction in fly. An important novel feature of our model is that it explicitly infers the phylogenetic distribution of functional target sites, independently for each miRNA. This allows us to infer species-specific and clade-specific miRNA targeting. The method has no tunable parameters, and can be readily applied to update the predictions as more genomes are being sequenced and more miRNAs discovered. Currently, we are working in collaboration with several experimental groups on the discovery of targets for specific miRNAs, by combining mRNA expression studies with miRNA target predictions. Examples of systems that we have been studying are the let-7 miRNA-dependent regulation in Caenorhabditis elegans (collaboration with Helge Grosshans, Friedrich-Miescher Institute), the regulation dependent on embryonic miRNAs in mouse (collaboration with Witek Filipowicz, Friedrich-Miescher Institute), metabolic regulation depending on the pancreas-specific miR-375 (collaboration with Markus Stoffel, ETH). A more global approach to the experimental identification of miRNA targets is to identify the mRNAs contained in immunoprecipitates of the Argonaute proteins. We recently performed such a study in collaboration with Tom Tuschl's group (The Rockefeller University), and we found that different Argonaute proteins tend to associate with the same mRNAs, as do variants of the TNRC6 protein, which is a component of the miRNA-containing P bodies [3].

Determinants of miRNA target site specificity

Reliable target predictions also enable the discovery of further determinants of miRNA targeting specificity. For instance, we showed that in long human 3' UTRs, miRNA target sites occur preferentially near the start and near the end of the 3' UTR [2]. These sites reside in sequence environments with strong nucleotide compositional biases, enriched in A and T nucleotides and depleted in C and G nucleotides. By further studying microarray expression data from miRNA over-expression experiments and siRNA off-targeting experiments, we find that similar sequence biases hold for sites whose functionality was assessed experimentally. Our aim is to discover further determinants of miRNA function using these types of data, and to then incorporate these into improved target prediction tools.

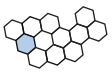
In the coming years we will continue to combine computational and experimental approaches to study both the mechanisms of miRNA-dependent regulation as well as the specific targets of individual miRNAs.

Discovery of other classes of small regulatory RNAs



Distribution of piRNAs along two divergently transcribed regions on mouse chromosome 17 and the corresponding distribution of piRNAs sequenced from the orthologous human regions. The overall structure of the piRNA loci is conserved between mouse and human, although the piRNA sequences themselves are not.

The second abundant class of small RNAs that have been discovered so far are the Piwi-protein-associated small RNAs (piRNAs). Sequences of small RNAs from testis samples revealed that at the onset of meiosis a population of 26-30 nucleotide long RNAs appears, at an overall abundance surpassing that of miRNAs [4,5,6]. In fly, piRNAs are expressed both in ovaries [7] and testes, while in mammals, piRNAs have so far been found only in testes. Computational analysis indicated that piRNAs are processed from long primary transcripts (see Figure), but that in contrast to miRNAs, the processing does not appear to involve double-stranded intermediates [4,5,6]. The precise mechanisms of piRNA transcription and processing are the subject of intense study in our as well in other groups, as is their function. Evidence from fly suggests that at least part of the piRNA function is in the silencing of repeat elements [7].



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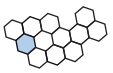

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Core Program
Structural Biology & Biophysics

Introduction

The ultimate understanding of biological function rests on the detailed description of biomolecular interactions in terms of structure, physical forces, and the resulting dynamics. Enormous advances have been made during the last decades in the techniques that visualize the three-dimensional structure and quantify the dynamics of cellular components down to the atomic level. It is the goal of the Core Program, Structural Biology and Biophysics‘ to apply and where possible further develop these structural and biophysical techniques in order to understand at all levels the interactions that make up the dynamical network of a living system.

The structural techniques applied range from light, electron and scanning microscopy over X-ray crystallography to NMR spectroscopy. Biophysical methods encompass many different time-resolved spectroscopic techniques such as laser-flash and stopped-flow optical spectroscopies, thermodynamic analytical methods such as ITC and DSC microcalorimetry, NMR imaging and in vivo spectroscopy. Using rigorous combinations of these techniques, we are in a unique position to determine the structure of biological matter at all length scales, from atomic detail over entire cells to small animals, to analyze and quantify dynamical changes starting from picosecond time scales to very long periods, and to determine the energetics and thermodynamics of biomolecular interactions at very high precision.

It is the clear perspective of the Core Program ‚Structural Biology and Biophysics‘ that the understanding of biological function goes beyond a pure static, structural description and that additional information is needed from other biophysical techniques. In all of the research areas described below, a very strong interplay exists between structure determination and biophysics that is being used for the characterization of the dynamics and stability of proteins and their interactions. Strong synergisms also exist with all the other focal areas of the Biozentrum since many of the systems are shared projects with other biochemically or biologically oriented groups.

Research Groups

The core program comprises six senior research groups headed by Ueli Aebi (electron and force microscopy), Andreas Engel (electron and force microscopy), Stephan Grzesiek (NMR spectroscopy), Thomas Kiefhaber (biophysics of protein folding), Tilman Schirmer (x-ray diffraction) and Joachim Seelig

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(membrane biophysics and NMR). In 2007 Thomas Kiefhaber moved to the Technical University in Munich after 14 years at the Biozentrum. We thank Thomas for his many outstanding contributions to the Core Program as a researcher, teacher and colleague.

Additional junior or independent project groups are headed by tenure track assistant professor Dagmar Klostermeier (single molecule fluorescence of molecular motors), non-tenure track assistant professor Olga Mayans (x-ray diffraction), Titularprofessor Anna Seelig (selectivity and kinetics of membrane transport), guest scientist Prof. Konstantin Pervushin (NMR spectroscopy), PD Birthe Fahrenkrog (structural biology of the nuclear pore complex), PD Dimitri Fotiadis (electron and force microscopy of membrane proteins), PD Heiko Heerklotz (lipid biophysics), PD Cora Schoenenberger (structural biology of actin) and PD Jörg Stetefeld (x-ray diffraction). The core program receives generous support by the M.E. Müller Foundation via the fully integrated M.E. Müller Institute for Structural Biology (research groups Ueli Aebi and Andreas Engel). These two group leaders are also project leaders in the National Centers of Competence in Research (NCCR) programs “Nanoscale Science“ (UA and AE) and “Structural Biology” (AE).

Highlights

A unique opportunity to ensure the long-term future of electron microscopy in Basel at the highest level has been provided in 2006 by the approval of the SystemsX glue project C-CINA (Center for Celluar Imaging and NanoAnalytics) headed by Andreas Engel. It is planned that the Engel group will relocate to the C-BSSE building as a contribution of the Biozentrum to the SystemsX initiative. As part of the C-CINA approval, funding for a successor of Engel (retiring in 2010) has been granted by the University (former Guggenheim position). As such Engel and his successor shall remain part of the Core Program Structural Biology and Biophysics.

In 2006 Dimitri Fotiadis successfully passed his habilitation.

In 2006 Jörg Stetefeld left the Biozentrum to accept a Canada Research Chair in Structural Biology at the University of Manitoba, Canada.

In 2006 Heiko Heerklotz left the Biozentrum to accept an Associate Professorship at the Leslie Dan

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Core Program
Structural Biology & Biophysics

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During 2004–2006 Joachim Seelig was Chairman of the 'Council of Scientists', of the Human Frontier Science Program Organisation. Since 2006, he is treasurer of the Human Frontier Science Program Publishing.

Joachim Seelig held the 'van Deenen lecture' at the 48th Conference on the Bioscience of Lipids at Turku, Finland, Sept. 6, 2007.

In 2007 Anna Seelig was appointed Titularprofessor of the University of Basel.

The EMBO Practical Course 'Structure determination of biological macromolecules by solution NMR' was organized by Stephan Grzesiek from July 6 to 13, 2007 at the Biozentrum.

Teaching

The knowledge of structural principles of biomacromolecules and the understanding and quantitative application of biophysical methods are prerequisites for success in all areas of biomolecular research. In addition to the specific research, the Core Program's teaching contributes fundamentally to the Biozentrum's Biology curriculum. Structural biology and biophysical theories together with the relevant techniques are being taught to all students of the Biozentrum at various levels by all members of the Core Program. Undergraduate lectures are in part also attended by students of Pharmaceutical Sciences, Chemistry, Physics and Nano-Science.

Structure-based functional analysis of cellular nanomachines by a hybrid methods' approach

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The long-term goal of the lab is to mechanistically understand the formation, functioning and turnover of cellular nanomachines, and more generally, supramolecular assemblies. This goal is achieved by a structure-based hybrid methods' approach employing light, electron and scanning probe microscopies, X-ray crystallography, biophysics, biochemistry, and molecular cell biology. Our problem-driven research focuses on (1) cytoskeleton structure, assembly and mechanics; (2) the nuclear pore complex and nucleocytoplasmic transport; and (3) developing and implementing novel optical and mechanical nano-sensors and nano-actuators for local diagnostics and therapy by minimally invasive interventions. In addition to the research directed by U. Aebi, the lab hosts two groups headed by independent project leaders, PD Dr. Cora Schoenenberger and PD Dr. Birthe Fahrenkrog, which have in part their own funding and collaborate on various projects with members of the Aebi group. The progress of their projects is presented in independent reports. Following is a selection of recent findings made in the Aebi group.

Deciphering the assembly mechanism of vimentin intermediate filaments by near-uv circular dichroism

Dr. Sofia Georgakopoulou is using near-uv circular dichroism (CD) for identifying distinct "signatures" of human vimentin's various stages of association during its assembly into intermediate filaments (IFs). CD probes the distinct near-field interactions of a protein's aromatic amino-acids occurring within its different oligomeric/polymeric states. More specifically, assembly of vimentin A11 tetramers into IFs was induced and the spectral changes were recorded during several hours. The observed signal changes were compatible with a radial compaction of the tetrameric building blocks prior to their assembly into IFs. Also, after storage at 4°C the observed CD signal change indicated breakage of the filament structure into shorter units and loss of some IF-specific tetramer conformations. These results were complemented by measurements of several mutant vimentin variants. In contrast to x-ray crystallography or electron microscopy, near-uv CD spectroscopy allows for continuous monitoring of the overall conformational state of a protein oligomer/polymer in a non-invasive way and under physiological conditions.

Assessing the tensile properties of single desmin intermediate filaments

Within muscle fibers, desmin intermediate filaments (IFs) are major constituents of the extra sarcomeric

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cytoskeleton. However, their contribution to the mechanical properties of myocytes has remained elusive. Dr. Laurent Kreplak has developed an experimental approach to measure the extensibility and the tensile strength of *in vitro* reconstituted desmin IFs adsorbed to a solid support. The tip of an atomic force microscope (AFM) was used to push on single filaments perpendicular to the filament axis. The torque of the AFM cantilever was monitored during the pushing events to yield an estimate of the lateral force necessary to bend and stretch the filaments. Desmin IFs were stretched up to 3.4 fold with a maximum force of ~3.5 nN. Fully stretched filaments exhibited a much smaller diameter than native IFs, i.e. ~3.5 nm compared to 12.6 nm, both by AFM and electron microscopy. Moreover, we combined the morphological and lateral force data to compute an average stress-strain curve for a single desmin filament. The main features were a pronounced strain-hardening regime above 50% extension and a tensile strength of at least 240 MPa. Because of these non-linear tensile properties, desmin IFs may dissipate mechanical energy and serve as a physical link between successive sarcomeres during large deformation.

Dissecting the nanomechanical basis of selective gating by the nuclear pore complex

The nuclear pore complex (NPC) regulates cargo transport between the cytoplasm and the nucleus. Dr. Roderick Lim set out to correlate the governing biochemical interactions to the nanoscopic responses of the phenylalanine-glycine (FG)-rich nucleoporin domains, which are involved in attenuating or promoting cargo translocation. He found that binding interactions with the transport receptor, karyopherin-β1, caused the FG-domains of Nup153 to collapse into compact molecular conformations. This effect was reversed by the action of RanGTP, which returned the FG-domains into a polymer brush-like, entropic barrier conformation. Similar effects were observed in Xenopus oocyte nuclei *in situ*. Thus, we anticipate that the reversible collapse of the FG-domains may play a significant role in regulating nucleocytoplasmic transport.

Towards early detection of osteoarthritis in mouse and human articular cartilage by indentation-type atomic force microscopy

Despite the high prevalence of osteoarthritis (OA) in people over 50, there are currently no treatments available that allow for halting or reversing the disease. One major limitation in the development of

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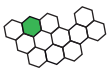

efficient treatments of OA is the lack of sensitivity of current diagnostic tools. The ability of probing early onset of OA, i.e., when the disease may still be stopped or cured, may lead to more rational treatment strategies and to the development of effective drugs. Dr. Martin Stolz has employed indentation-type atomic force microscopy (IT AFM) to directly probe cartilage structure/function relationships at the molecular scale. More specifically, he followed age-related structural and functional changes in the articular cartilage of wild-type mice. Next, he documented age-dependent OA-like changes in collagen 9 knock-out mice. Finally, he obtained first IT AFM results on human OA cartilage. Most significantly, the early changes in aging and OA were only detectable at the nanometer scale, but not at the larger scales. Our findings propose the use of IT AFM for unraveling the early structure/function changes in the development of OA *ex vivo*. Ultimately, IT AFM may be employed directly *in situ*, for example, by an arthroscopic intervention.

Last but not least, Marko Loparic, an MD-PhD student is employing IT AFM for mechanical testing of tissue engineered cartilage (TEC), in particular, functional testing of the proteoglycan moiety in TEC. Longer-term, IT AFM may become a useful clinical tool for effective repair of diseased cartilage, to stimulate transplanted cartilage, and to design and produce TEC exhibiting long-term mechanical stability and biocompatibility.

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Baer, H., Muecke, N., Ringler, P., Muller, S. A., Kreplak, L., Katus, H. A., Aebi, U. & Herrmann, H. (2006). Impact of disease mutations on the desmin filament assembly process. *J Mol Biol* 360, 1031-42.

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Membrane protein structure and function

Biological membranes are vital components of all living systems, forming the boundaries of cells and their organelles. They consist of a lipid bilayer and of embedded proteins, which are nanomachines that fulfill key functions such as energy conversion, solute transport, secretion, and signal transduction. Lipids and membrane proteins form domains, which can vary and adapt to the functional state of the cell. Large solubilized, membrane bound complexes can be studied by cryo-electron microscopy (EM) and single particle analysis. Two-dimensional (2-D) crystals of purified membrane proteins reconstituted in the presence of lipids provide a close to native environment and allow the structure and function of membrane proteins to be assessed. 3-D information is extracted from these 2D crystals by electron crystallography at atomic resolution. The atomic force microscope (AFM) is used to study membrane protein surfaces at sub-nanometer resolution and in buffer solution, providing information about their conformational variability at the single molecule level. Combining these methods we continue to study different aquaglyceroporins, ATPases, channels and G-protein coupled receptors. In pursuit of the goal to produce and analyze 2D crystals of different membrane proteins, we have invested a significant effort to express different membrane proteins in various expression systems, and have pushed the methodology of electron crystallography. In parallel, we use the STEM for mass determination and single particle imaging to acquire information of biological relevance.

STEM (in collaboration with G. Cornelis, A. Economou, R. Glockshuber, R. Jahn)

The simplicity of dark-field image formation in the scanning transmission electron microscope (STEM), and the quantitative nature of these images provide a basis to collect structural information at the single complex level and to determine the mass of protein complexes. For this reason and because the Basel STEM is the only one operating in Europe, we are engaged in a number of collaborations. Superb images of hybrid tip structures of Yersinia injectosomes have clarified the structural arrangement of LcrV, and allowed the function of YscU as an essential component of the export machinery to be confirmed. Mass measurements have been decisive in determining the 3D structure of HrcN, the type III secretion system protein translocase, and of ClyA, a torpedo-shaped bacterial toxin by cryo-electron microscopy (EM). Mass-per-length measurements have contributed to the understanding of the desmin filament assembly in disease, and mass determina-

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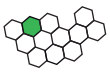

tion of synaptic vesicles provided the necessary link to assemble a wide range of data into a stoichiometric model of this important organelle. Occasionally the STEM gives us headaches, because the instrument has been developed in the seventies and the company does not exist anymore. Yet, the investment in maintaining the Basel STEM is worthwhile, as documented by its regular contributions to important biological questions.

Aquaporins (in collaboration with P. Kjellboom and R. Kaldenhoff)

We keep working on water channels, because aquaporins fulfill a key function in maintaining the homeostasis of all organisms. AQP2, the regulated water channel of the renal collecting duct, is regulated by vasopressin that activates the vasopressin receptor V2R, which in turn leads to phosphorylation of AQP2. We are after the structure of the phosphorylated and dephosphorylated AQP2 tetramer, and would like to identify the protein that binds to the phosphorylated form and induces its transport to the apical membrane. We study SoPIP2;1, a major aquaporin of the plant plasma membrane. We have obtained 2D crystals of SoPIP2;1 that diffract to 2Å. We are currently collecting a full data set to solve the structure, and perform experiments on the water transport activity of different SoPIP2;1 mutants.

Rhodopsin (in collaboration with K. Palczewski)

To confirm the functional oligomeric state of rhodopsin, different solubilization conditions with n-dodecyl-β-D-maltoside (DDM) n-tetradecyl-β-D-maltoside (TDM), and n-hexadecyl-β-D-maltoside (HDM) were used to produce higher oligomers. Negative stain EM showed the rhodopsin oligomers to increase in size with milder detergents – worm like oligomers akin to the rows of dimers in native disk membranes were found for HDM. At the same time the capacity of rhodopsin to activate transducin increased more than ten fold when HDM was employed for solubilization instead of DDM.

ClyA (in collaboration with U. Grauschopf and R. Glockshuber)

ClyA, a pore-forming toxin from virulent *Escherichia coli* and *Salmonella enterica* strains was analyzed biochemically and its structure determined by cryo-EM. We showed that the intrinsic hemolytic activity of ClyA is independent of its redox state, and that the assembly of both reduced and oxidized ClyA to the ring-shaped oligomer is triggered by contact with lipid or detergent. A rate-limiting conformational transition in membrane-bound ClyA monomers precedes their assembly to the functional pore. We obtained a 3D model of the detergent-induced oligomeric complex at 12 Å resolution by combining cryo- and negative stain EM with mass measurements by STEM. The model reveals that 13 ClyA monomers assemble into a cylinder with a hydrophobic cap region, which may be critical for membrane insertion.

V-ATPase (in collaboration with R. Milligan)

Bovine V-ATPase from brain clathrin-coated vesicles was investigated by cryo-EM and single particle analysis. Our studies revealed the flexibility of the central linker region connecting V1 and VO. As a consequence, the two sub-complexes were processed separately and the resulting volumes were merged to yield the first 3D map of a V-ATPase from cryo-EM data. The overall resolution was estimated to 34 Å. Our 3D reconstruction shows a large peripheral stalk and a smaller, isolated peripheral density, suggesting a second, less well-resolved peripheral connection. The 3D map reveals new features of the large peripheral stator and of the collar-like density attached to the membrane domain. Our analyses of the membrane domain indicate the presence of six proteolipid subunits. In addition, we could localize the VO subunit a, which is flanking the large peripheral stalk.

HrcN (in collaboration with A. Economu and H. Stahlberg)

The structure of HrcN was determined by cryo-EM. The specialized type III secretion (T3S) apparatus of pathogenic and symbiotic Gram-negative bacteria comprises a complex transmembrane organelle and an ATPase homologous to the F1-ATPase β subunit. The T3S ATPase HrcN of *Pseudomonas syringae* associates with the inner membrane, and its ATP hydrolytic activity is stimulated by dodecamerization. The structure of dodecameric HrcN (HrcN12) was determined to 16 Å by cryo-EM. HrcN12 comprises two hexameric rings that are probably stacked face-to-face by the association of their C-terminal domains. It is 11.5±1.0 nm in diameter, 12.0±2.0 nm high and has a 2.0 to 3.8 nm wide inner channel.

VDAC

Voltage-dependent anion channels (VDACs) transport nucleotides, ions and metabolites across the outer mitochondrial membrane (OMM). We have isolated, characterized and imaged OMMs of po-

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tato tubers. SDS–PAGE and mass spectrometry of OMMs revealed the presence of the VDAC isoforms POM34 and POM36, as well as the translocase complex of the OMM. Atomic force microscopy of freshly isolated OMMs demonstrated the existence of VDAC monomers to tetramers, hexamers and higher oligomers, and the spatial arrangement of VDACs within the native membrane. The observed oligomerization has implications for the regulation of VDAC function, for the binding of hexokinase and creatine kinase to the OMM and for mitochondria-mediated apoptosis.

Amino acid and peptide transporters (in collaboration with M. Palacin and H. Daniel)

During the last three years, we have focused on the structure and function of amino acid and peptide transporters within the "European Genomics Initiative on Disorders of Plasma Membrane Amino Acid Transporters" (EUGINDAT) of the 6th EC Framework Program. Over 20 selected prokaryotic and eukaryotic target transporters with high identity and homology to disease-related human amino acid and peptide transporters were functionally and structurally characterized. We managed to grow 2D crystals of three amino acid and two peptide transporters as well as the first 3D crystals of a peptide transporter. So far, we have published the functional, biochemical and structural characterization of one amino acid and one peptide transporter; further manuscripts are in preparation.

Membrane protein expression

The commonly used eukaryotic expression systems - yeast, baculovirus/insect cells (Sf9) and Semliki Forest Virus (SFV)/mammalian cells – were explored for the expression of different eukaryotic membrane proteins. Expression levels, quality, biological activity, localization and solubility of all expressed proteins are compared to identify the advantages of one system over the other. SFV-transfected selected mammalian cell lines provide the closest to native environment for the expression of mammalian membrane proteins, and they exhibited the best overall performance. But depending on the protein, Baculovirus-transfected Sf9 cells performed as well as mammalian cells. The lowest expression levels for the proteins tested were obtained in yeast. We have expressed GPCRs (vasopressin type-2 receptor (V2R), α-factor receptor (Ste2p), and the human P2Y2 in Sf9 cells, HEK cells and BHK cells. The amounts of functional receptors produced and purified did not suffice for structural studies so far.

2D crystallization technology (2DX) (in collaboration with G. Vandergoot, EPFL)

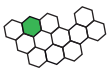
A crystallization robot was developed to assemble 2D crystals by cyclodextrin-driven detergent removal. Several membrane proteins have been reconstituted and/or crystallized. First large 2D crystals of aerolysin were obtained in collaboration with G. Vandergoot, EPFL after initial crystallization conditions were determined by dialysis. The accurate adsorption of detergent molecules by cyclodextrin opens possibilities to improve the crystallization kinetics that are to be explored. In this respect, precise knowledge of the detergent concentration is important. We have developed a robust method to measure this parameter efficiently by a contact angle measuring apparatus.

Image processing

A large effort is invested in making robust, portable and easy to use software available to the electron crystallography community. This effort has led to iplt (image processing library tools), which is a flexible and extendable, open-source cross-platform framework for image processing of electron microscope images. A major part of this development concerns the processing of electron diffraction patterns, another part improves the processing of images from 2D crystals.

Future direction

We continue to address the major problems in protein expression by systematically screening for well behaved human membrane proteins. We anticipate to finally solve one or even two structures by using data collected and software developed in house. In view of progress in SystemsX, we will enhance our collaborations with the Swiss research community, offering STEM, AFM and cryo-electron microscopy to assess the structure of macromolecular complexes. In particular, we will build up the center for Cellular Imaging and NanoAnalytics (CINA), which will comprise a 300 kV cryo-TEM with energy filter for electron tomography, a 200 kV cryo-TEM for single particle analysis, STEM and a system for serial block face imaging.



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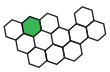

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Analysis of nuclear pore complex proteins in nuclear transport and other cellular processes

Research

Our research focuses on the functional role of distinct nuclear pore complex proteins (nucleoporins) in nuclear transport as well as other cellular processes, such as cell division. The nuclear pore complex (NPC) is the sole gateway between the nucleus and cytoplasm of interphase eukaryotic cells, and it mediates all trafficking between these two cellular compartments. NPCs are composed of about 30 different proteins, known as nucleoporins, and defects in nuclear transport as well as dysfunction of nucleoporins have been detected in distinct human diseases, such as cancer and autoimmune diseases. Based on the structure of the NPC, we are aiming to gain a more rational understanding of the functional role of individual nucleoporins for NPC structure and function.

Analysis of nucleoporin function

The NPC is composed of about 30 nucleoporins, which are typically characterized by a distinct domain organization. A common feature of about one third of those nucleoporins is the presence of variable FG (phenylalanine-glycine)-repeat motifs, which are thought to play critical roles in the interaction between nucleoporins and soluble transport receptors as they traverse the NPC. In order to better understand the role of FG-repeat and other nucleoporin domains for NPC structure, function and nuclear transport, we have previously mapped the domain topology of the vertebrate nucleoporins Nup153, Nup214 and Nup62 for different nuclear transport states as well as in response to chemical effectors, such as Ca2+ and ATP by immuno-EM using *Xenopus* oocyte nuclei (in collaboration with K. Ullman, Huntsman Cancer Institute, University of Utah, Salt Lake City, and M. Powers, Emory University School of Medicine, Atlanta). We could show that Nup153, Nup214 and Nup62 are anchored to the NPC by their non-FG repeat domains, whereas their FG-repeat domains are flexible and mobile within the NPC (Fahrenkrog *et al*., 2002; Fahrenkrog and Aebi, 2003; Paulillo *et al*., 2005; Paulillo *et al*., 2006; Schwarz-Herion *et al*., 2006). The actual location of the FG-repeat domains of Nup153 and Nup214 thereby changes in a nuclear transport-dependent manner, consistent with a role of FG-repeat domains in nuclear transport (Paulillo *et al*., 2005). Moreover, the FG-repeats of Nup153 and Nup214 appear sensitive to chemical changes within the near-field environment of the NPC, in particular to increasing concentrations of divalent cations, which

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supports the notion that FG-repeat domains are natively unfolded molecules (Paulillo *et al*., 2006).

The main function of the NPC is to mediate the trafficking between the nucleus and cytoplasm. Despite the fact that the key factors that play a role in nucleocytoplasmic transport have been identified in the last couple of years, the question as to how the NPC on one hand mediates translocation of specific cargo while on the other hand excludes non-specific cargo has remained elusive. To address this question, we have, in collaboration with R. Lim and U. Aebi (Biozentrum Basel), tethered the recombinantly expressed FG-repeat domain of Nup153 to nanometer-sized gold dots to study their behavior on a nanoscopic scale. By employing atomic force microscopy (AFM), we found that the FG-repeat domain of Nup153 behaves like a polymer brush, indicating that FG-repeats in the NPC act as entropic barrier that facilitate nuclear transport of specific cargoes, but prevent the passage of macromolecules that are not able to interact with the FG-repeats (Lim *et al.*, 2006). Furthermore, the FG-repeat domain of Nup153 collapses into a more compact molecular conformation upon binding of the nuclear import receptor importin ß, and this collapse is reversed to the brush-like conformation by the action of the small GTPase Ran, when loaded with GTP as shown by AFM and immuno-EM (Lim *et al*., 2007). Together these studies provided significant new insights into our understanding of the gating mechanism underlying NPC translocation.

After these extensive studies in particular on the FG-repeat domain of Nup153, we have now switched our interest on the function of the non-FG-repeat domains of Nup153, and hereby especially its role in cell division.

Elucidating the molecular mechanism underlying yeast apoptosis

A second research interest of our group is apoptosis in yeast. Apoptosis is a form of programmed cell death that is crucial for the development and maintenance of multicellular organisms and in the past few years it became evident that such a death program also exists in unicellular organisms, such as the yeast Saccharomyces cerevisiae. In this context, our studies on the yeast nucleoporin Nic96p have led us to the identification of the nuclear serine protease Nma111p, which we found to promote apoptosis in yeast (Fahrenkrog *et al*., 2004). In collaboration with F. Madeo (University of Graz, Austria) we have then identified Bir1p, the only inhibitor-of-apoptosis (IAP) protein found in the yeast genome, as substrate of Nma111p (Walter *et al*., 2006). Moreover, we could show that Bir1p is degraded by Nma111p *in vivo*, which enhances apoptosis, whereas overexpression of Bir1p inhibits cell death. Bir1p was originally identified as component of the yeast chromosome passenger complex and as such acting in chromosome segregation and cytokinesis. Bir1p undergoes several posttranslational modifications, such as phosphorylation and sumoylation, and we are therefore now, besides studying the interplay between Nma111p and Bir1p in more detail, analyzing the importance of these posttranslational modifications of Bir1p for its anti-apoptotic activity.

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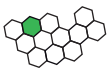

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High resolution NMR of biological macromolecules

The scientific goal of the group is the application and development of high-resolution Nuclear Magnetic Resonance (NMR) methods for the elucidation of structure, function, and dynamics of biological macromolecules.

Our work is directed towards problems in structural biology where NMR can give unique information:
1. the determination of biomolecular structures and interactions which are difficult to obtain by other methods. A special emphasis is put onto biomolecular pathogens, not completely folded structures, and membrane-associated proteins. 2. the study of macromolecular complexes where NMR yields information on interaction surfaces, the strength of the interaction and the dynamics, and thereby offers insights into biomacromolecular mechanisms 3. the study of forces that are relevant for macromolecular folding.

Structural Projects

Hexim1

The positive transcription elongation factor b (P-TEFb) is essential for transcriptional elongation in human cells. It comprises one cyclin (T1 or T2) and one kinase, cyclin-dependent kinase 9 (CDK9), which phosphorylates the carboxyl-terminal domain of RNA polymerase II. During HIV infection, a highly specific interaction among cyclin T1, the HIV protein Tat, and the transactivation response (TAR) element RNA leads to the productive transcription of the viral genome. Recently, the human protein Hexim1 has been shown to also be part of the P-TEFb complex and to compete with Tat/TAR binding.

We have now solved the solution structure of Hexim1. It consists of a homodimeric parallel coiled coil and an adjacent helix that is preceded by a highly flexible N-terminal region. NMR titration and mutagenesis studies have revealed the binding interface to Cyclin T1. Based on isothermal calorimetry, gelfiltration, and fluorescence data, the stoichiometry of the complex and its affinity have been determined (in collaboration with M. Geyer, MPI Mol. Physiol., Dortmund).

T-cadherin

Human T-cadherin is a receptor for low-density lipopoliproteins and adiponectin involved in angiogenesis and cell growth and migration. In contrast to the classical epithelial cadherin, T-cadherin does not have a tryptophan residue at position 2 and does not contain an HAV-motif, which has been proposed as a determinant for tissue specificity in classical cadherins. We have now solved the structure of the first

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domain of human T-cadherin (TCAD1) by solution NMR. Similar to the other characterized cadherin domains, TCAD1 adopts a β-barrel structure with a Greek folding topology, but in contrast to the latter has an additional stable β-sheet around I2. EM and solid-phase binding data of T- and E-cadherin constructs encompassing all 5 cadherin repeats showed that T-cadherin has a much lower adhesive capacity than classical cadherins (in collaboration with J. Engel, Biozentrum).

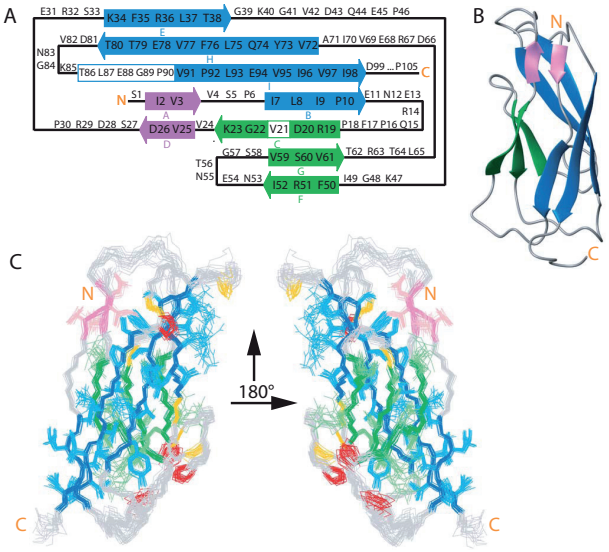

Figure 1: NMR structure of TCAD1.

Rantes-CCR5 interactions

The chemokine RANTES is a natural ligand of CCR5, one of the major HIV-1 coreceptors. It is secreted as part of the immune response to HIV-1 and inhibits infection by CCR5-dependent (R5) HIV-1 isolates. We have investigated the interaction of RANTES with several peptides derived from the extracellular parts of CCR5 by heteronuclear NMR spectroscopy in aqueous solution. We have shown that a peptide comprising the first 25 amino acids of the CCR5 N-terminal domain and sulfated at the Y10- and Y14-sidechains binds with micromolar affinity exclusively to the monomeric form of RANTES, but not to its dimeric form. Peptides derived from the CCR5 extracellular loops ECL1-3 showed only very moderate and mostly nonspecific binding. Chemical shift mapping of the interaction of the sulfated N-terminal peptide reveals a binding surface that largely overlaps with the dimer interface and is strongly positively charged, providing a rationale for the exclusive binding of the monomer to the peptide and the requirement of the negative sulfate groups. The binding surface also largely overlaps with the segments that were previously identified as crucial for HIV blockade by peptide scanning and mutagenesis studies. These data offer new insights into the structure-function relation of the RANTES-CCR5 interaction and may be helpful for the design of novel HIV-1 inhibitors.

We have extended this project with the very ambitious goal to also characterize full CCR5. We have now obtained good expression of CCR5 in the Baculovirus/insect cell system. The purified CCR5 is able to recognize RANTES. The ultimate goal is to obtain structural information on the RANTES/CCR5 interaction by solution NMR, solid state NMR or X-ray crystallography (in collaboration with P. Lusso, DIBIT, Milan).

DgrA – a cyclic diguanosine monophosphate receptor

The bacterial PilZ domains are targets for signaling via cyclic di-GMP, which causes a behavioral change of bacterial communities between motile single cells and multicellular ensembles that colonize surfaces. Understanding this mechanism may offer new routes to treatment of bacterial infections. U. Jenal, Biozentrum, has identified the PilZ homolog PA4608 as a new receptor for cyclic di-GMP. We have carried out an NMR analysis of the transition between free and c-di-GMP-bound forms of PA4608. The NMR data show that PA4608 undergoes a large structural change upon ligand binding. The binding surface was determined by chemical shift mapping. We are currently working on the structure determination of the full cyclic-di-GMP-PA4608 complex (in collaboration with U. Jenal, Biozentrum).

Structure, dynamics and interactions of $^{13}C,^{15}N$-labelled lipopolysaccharides in membrane-bound form

Lipopolysaccharides (LPS, endotoxins) are major components of the outer membrane of Gram-negative bacteria, which makes them prime targets for recognition by the immune system via specific endotoxin receptors. In small amounts, LPS provoke a beneficial stimulation of the immune system. However, in larger amounts they lead to septic (endotoxic) shock. A detailed molecular description of the early recognition events of LPS by their receptors is of great medical interest and essential for the understanding of pro-inflammatory processes of the innate immune system.

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Investigations of the three-dimensional and even the primary chemical structures of LPS molecules under natural conditions are hampered by the high intrinsic tendency of these zwitterionic and amphipathic compounds to form larger, stable aggregates in water. We have obtained ^{13}C,^{15}N-labelled LPS from a deep rough Re-mutant of *E. coli* and have been able to solubilize this LPS in the form of DHPC detergent micelles. The solubilized LPS yields high quality NMR spectra. We have been able to obtain structural information on LPS by NOEs and residual dipolar couplings and determined the structure of the first four sugars containing the endotoxic recognition motif (in collaboration with U. Zähringer, Forschungszentrum Borstel).

Minicollagen cysteine-rich domains – a model for protein fold evolution

Minicollagens from *Hydra* are the smallest known collagens to date. They are found in the walls of nematocysts, which are explosive organelles formed from a post-Golgi vesicle in *Hydra*, jellyfish, corals and other *Cnidaria*. Nematocyst walls are stabilized by a collageneous matrix, which depends on disulfide crosslinking to withstand an extreme osmotic pressure of up to 150 bar. The *Hydra* capsule wall mainly consists of the proteins minicollagen and NOWA. Both proteins contain highly homologous cysteine- and proline-rich domains, called minicollagen cysteine rich domains (CRDs). Minicollagens are expressed as soluble precursor forms with intramolecular disulfide bonds in their CRDs. In nematocyst development a wall hardening occurs, during which the intramolecular CRD disulfide bonds are most likely reshuffled to intermolecular bonds, thereby crosslinking molecules within the capsule wall.

In the last years, we have determined the structure of a number of these CRDs by modern heteronuclear and weak alignment NMR techniques. The aim of these studies is to understand how the different cysteine sequence patterns lead to different structural motifs, and possibly how the intermolecular disulfide reshuffling leads to the extremely stable nematocyst cell walls. The domains typically consist of 20-30 amino acids containing six cysteines and up to six prolines, which form hitherto unknown folds.

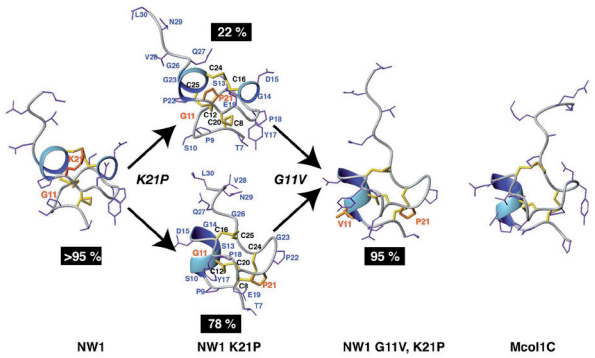

Figure 2: evolution of a protein fold by a single amino acid change.

Based on these studies we have been able to show that a global structural switch can arise from single amino acid changes in the CRDs. By applying a structure-based mutagenesis approach we could demonstrate that a CRD domain can interconvert between two natively occurring domain structures via a bridge state that contains both structures. A comparison of cnidarian CRD sequences leads us to believe that the mutations we introduced to stabilize each structure reflects the birth of new protein folds in evolution (in collaboration with S. Özbek, T. Holstein, University of Darmstadt, Germany and H.P. Bächinger, Oregon Health and Science University, Portland, USA).

NMR technique development

Structure and order of biomacromolecules from residual dipolar couplings

Recently the weak alignment of proteins in anisotropic liquid phases has become a tool to directly monitor average net orientations and order parameters of individual bonds by residual dipolar couplings (RDCs). The introduction of inert orienting media, such as strained polyacrylamide gels, has made it possible to obtain weak alignment and RDC information even under relatively harsh unfolding conditions. Our goal is to use RDCs for the quantitative description of unfolded or partially unfolded and also folded protein states. We have been able to make progress in this direction in several ways.

Residual dipolar couplings in short peptides reveal systematic conformational preferences of individual amino acids

We have systematically investigated the influence of amino acid substitutions X on the conformation of unfolded model peptides EGAAXAASS as monitored by RDCs. Fifteen single amino acid substitutions were investigated. The RDCs show a specific dependence on the substitution X that correlates to steric or hydrophobic interactions with adjacent amino acids. In particular, the RDCs for the glycine and proline substitutions indicate less or more order, respectively, than the other amino acids. The RDCs for aromatic substitutions tryptophane and tyrosine give evidence of a kink in the peptide backbone.

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RDCs for a substitution with the β-turn sequence KNGE differ from single amino acid substitutions. The results were compared to statistical models of unfolded peptide conformations derived from PDB coil subsets, which reproduce overall trends for $^{1}H^{N}$-^{15}N RDCs for most substitutions, but deviate more strongly for $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ RDCs. The outlined approach opens the possibility to obtain a systematic experimental characterization of the influence of individual amino acid/amino acid interactions on orientational preferences in polypeptides (in collaboration with M. Blackledge, IBS, Grenoble)

Direct Observation of Dipolar Couplings and Hydrogen Bonds across a β-hairpin in 8 M Urea

A detailed, quantitative description of the unfolded states of proteins at atomic resolution has been elusive due to enormous experimental and theoretical problems resulting from the huge number of degrees of freedom of an unfolded structural ensemble. In particular, direct long-range information has been extremely sparse. We have now been able to show that such long-range information can be obtained by NMR with high sensitivity and precision from H^{N}-H^{N} residual dipolar couplings (RDCs) and hydrogen bond (H-bonds) scalar couplings for an unfolded, perdeuterated (amide protonated) protein (urea-denatured ubiquitin at pH 2.5). Besides numerous sequential contacts, the RDCs reveal the persistence of native-like structure in ubiquitin's first β-hairpin. This native-like structure is confirmed by the direct detection of H-bonds via $^{h3}J_{NC'}$ H-bond scalar couplings as well as by chemical shifts, $^{3}J_{HNHA}$ couplings and relaxation rates (in collaboration with M. Blackledge, IBS, Grenoble).

High precision determination of the structure and order of folded proteins

We have determined a very large number (~1000) of long-range and short RDCs from different alignment media for perdeuterated folded protein G. Using solely these RDCs, we have now been able to obtain a *de novo* backbone structure of protein G. With a root mean square deviation of the backbone heavy atom positions of 0.4 Å relative to the 1.1 Å-resolution X-ray structure, this *de novo* structure is exceedingly well defined and yields particularly good hydrogen bond geometries (in collaboration with M. Blackledge, IBS, Grenoble)

Correlation of protein structure and dynamics to scalar couplings across hydrogen bonds

Hydrogen bonds (H-bonds) are essential for the stabilization of protein and nucleic acid secondary structure and often play a fundamental role in enzymatic reactions. Most of the evidence for hydrogen bonds in biological macromolecules is usually indirect e.g. from the spatial proximity of donor and acceptor groups. By NMR direct evidence for H-bonds can be provided in the form of scalar couplings, which connect magnetic nuclei on both sides of the hydrogen bridge. This effect can be used to "see" all three partners of a H-bond directly in an NMR experiment, i.e. the donor, the acceptor and the proton itself. The size of the couplings presents a quantitative measure for the geometry and - by the implicit experimental time averaging - dynamics of hydrogen bonds.

It has been under debate to what extent the behavior H-bonds is influenced by dynamics. We have carried out in-depth molecular dynamics (MD) simulations with various force fields on three proteins, i.e. ubiquitin, the GB1 domain of protein G and the SMN Tudor domain, for which experimental scalar couplings across backbone H-bonds and various high resolution X-ray structures are available. Theoretical average values for the couplings were calculated from the snap-shots of these MD simulations either by density functional theory or by a geometric parametrization. The results indicate that time averaging using explicit water solvation in the MD simulations improves significantly the agreement between experimental and theoretical values for lower resolution (2.1–1.8 Å) structures but not for a high-resolution (1.1 Å) structure. Hence, experimental H-bond couplings are compatible with a static, high-resolution structural model. This finding corrects earlier views held in the literature that dynamical averaging needs to be invoked to explain the behavior of hydrogen bonds in proteins.

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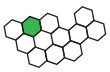

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NMR structural studies of flexible enzymes

Main research interests are the structure-dynamics investigation of flexible enzymes using high resolution NMR techniques. During the last year,we have studied the spatial structure and dynamical properties of a folding intermediate of the dimerization domain from the yeast transcription activation factor GCN4 (Leu zipper; LZGCN4). To achieve this, we have introduced a new line of NMR experiments fully optimized for spin state exchange applied to $^{1}H^{N}$, ^{15}N and $^{1}H^{\alpha}$,$^{13}C^{\alpha}$ moieties. We found that in a broad range of pH and buffer conditions the classical LZGCN4 coiled coil dimer is in a dynamic equilibrium with another distinct conformation (denoted here as x-form) and obtained complete assignment of the resonances stemming from the x-form. The LZGCN4 x-form is generally less structured in comparison with the classical GCN4-p1 coiled coil, but still retains a structured alpha-helical central core. This works paves the way for structural studies of unusual catalytical properties of zipper molecules recently discovered by the group of Prof. Gutta of the University of Zurich.

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Effects of Cholesterol on Membrane Structure and Thermodynamics

Cholesterol has a great variety of functions and consequences in biological membranes and its specific interactions with membrane lipids have been considered the key to the formation of functional domains in vivo, so-called lipid rafts. In previous years, we had shown that it is not justified to assume that such rafts could be isolated as detergent-resistant membrane fraction since detergents themselves can induce and promote the formation of cholesterol-dependent domains (e.g., Heerklotz, *Biophys. J*. 2002). The relevance of this effect, originally proposed on the basis of thermodynamic data, has been confirmed in vivo (van Rheenen *et al*., *EMBO J*. 2005) and by AFM of model membranes (Garner *et. al*., *Biophys. J*. 2008) and contributed to a new view and definition of rafts that is no longer based on DRM isolation (Pike, J. Lipid Res. 2006).

We have provided a detailed, quantitative explanation of detergent-induced domain formation by measuring non-ideal pair interaction energies in complex mixtures of phosphatidylcholine (PC), sphingomyelin (SM), cholesterol, and Triton (Tsamaloukas *et al*., 2006a). If the attractive interaction between cholesterol and SM (Tsamaloukas *et al*., 2006b) is overcompensated by thermal energy, the system may still mix homogeneously. Triton shows a strongly unfavorable interaction with cholesterol so that its addition shifts the balance in favor of demixing and may induce the separation of Triton (along primarily with PC) from cholesterol (with SM). The so-formed cholesterol-rich domains are then detergent-resistant.

These findings illustrate the need for a better understanding of cholesterol-lipid interactions in detergent-free systems. We have obtained a detailed thermodynamic comparison of the interactions of cholesterol with unsaturated PC and SM by using a new ITC assay utilizing cyclodextrin (Tsamaloukas *et al*., 2006b). The study revealed that the (up to fivefold) higher affinity of cholesterol to SM is caused by a substantial gain in enthalpy (and opposed by entropy).

Competing, largely incompatible descriptions have been published of binary mixtures of unsaturated PC (such as POPC) with cholesterol, claiming either a coexistence of two phases (liquid ordered and disordered) or a homogeneous mixture. We could provide important new insight be using a new method, pressure perturbation calorimetry, which measures the coefficient of thermal volume expansion (Heerklotz & Tsamaloukas, 2006). This quantity shows strongly enhanced values at compositions and temperatures that were referred to as the phase coexistence range

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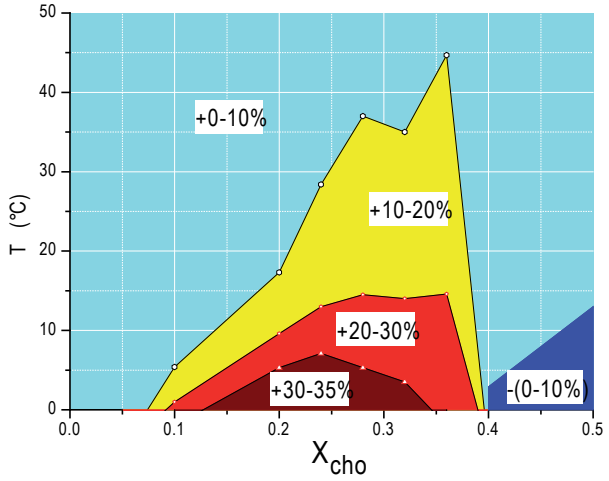
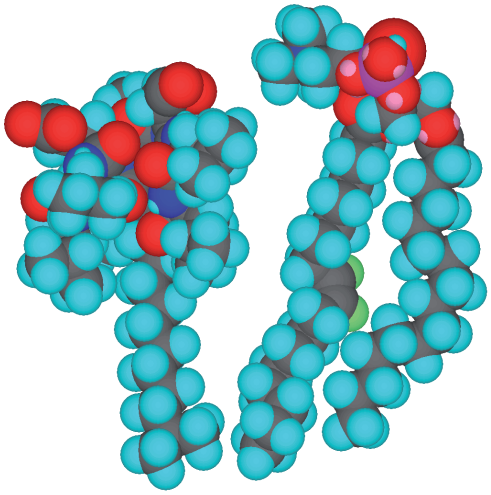


Relative changes of thermal volume expansion in mixtures of the lipid POPC with cholesterol (compared to pure POPC) as a function of cholesterol mole fraction, Xcho, and temperature, T. Adapted from (Heerklotz & Tsamaloukas, 2006).

but the detailed modelling reveals that the data obey the behaviour of a true phase separation only to a certain approximation. We found that at 2°C, each cholesterol molecule condenses the partial volume of ~3 PC molecules by ~-1.4%, which is quite substantial taking into account that liquids are virtually incompressible.

Interactions of Surfactants with Membranes

Further to the study of Trion interacting with complex lipid mixtures (Tsamaloukas *et al*., 2006a), we completed a series of publications on the interactions of the bacterial lipopeptide surfactin with lipid membranes (Heerklotz & Seelig, 2007).



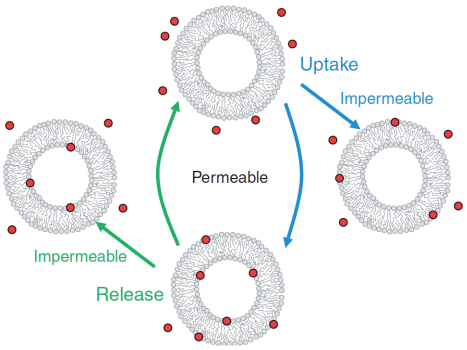
Schematical illustration of the lipopeptide surfactin (left) and the lipid POPC (right).

After studies of surfactin self association, membrane partitioning, and surfactin-induced changes in segmental order parameters of the lipid head groups and chains, we charaterized the ability of surfactin to induce membrane leakage and, finally, lysis by a combination of fluorescence assays, ITC, and NMR. The results suggest a sequence of mechanisms of membrane permeabilization with increasing peptide concentration, including bilayer rupture due to asymmetric insertion of peptide, stabilization of leaks by forming peptide-rich rim around its edge, and finally formation of mixed micelles.

We contributed to a study elucidating the effects of electrostatic interactions and counter ion binding to ionic surfactants on the phase behavior and detailed thermodynamic properties of lipid membrane solubilization (Keller *et al*., 2006b).

Development of Microcalorimetric Assays

We have developed the concept of detecting membrane binding and translocation of solutes by a combined application of uptake and release assays based on isothermal titration calorimetry. After extending the approach to insoluble (Tsamaloukas *et al*., 2006b) and charged (Keller *et a*l., 2006a) compounds, we published the detailed protocol and provided a evaluation script for download (Tsamaloukas *et al*., 2007).



Scheme illustrating the rationale of uptake & release assays. Both involve reverse transfers in case of permeable membranes but lead to different non-equilibrium states if the membrane is impermeable. Reproduced with permission from Macmillan Publishers Ltd: Nature protocols, (Tsamaloukas et al., 2007), copyright 2007.

Heiko Heerklotz

After introducing the new technique of pressure perturbation calorimetry to lipid membranes (Heerklotz and Seelig, Biophys. J. 2002), we have further explored and established new applications to membranes (Heerklotz & Tsamaloukas, 2006) and published the protocol and detailed technical description (Heerklotz, 2007).

Contributions to Projects in Structural Biology

In collaboration with the research groups of Prof. Schirmer and Prof. Jenal within the core program, we have shown by isothermal titration calorimetry that activation of the bacterial diguanylate cyclase PleD is paralleled by a tenfold stronger dimerization (Wassmann *et al*., 2007) but does not significantly affect c-di-GMP binding to the allosteric site (Paul *et al*., 2007).

We have also contributed to a project led by Prof. Mayans (Biozentrum) by showing that the fragment A168-A170 of titin binds differently truncated variants of the E3 ubiquitin ligase MuRF-1 with virtually the same affinity and enthalpy (Mrosek *et al*., 2007). For details of these projects, see presentations of the principal investigators within this report.

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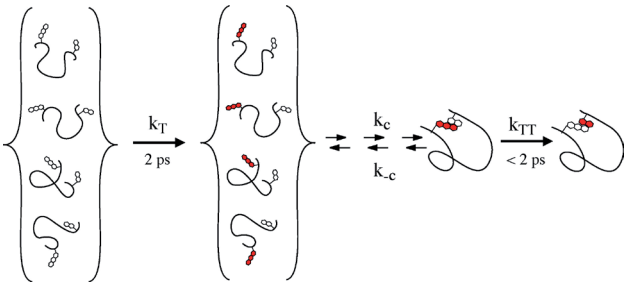
Mechanism and Dynamics of Protein Folding

Folding of a protein starting from the random coil is a spontaneous process *in vitro*. We use different approaches to gain insight into the mechanism of protein folding and into the dynamics of in different states of proteins. Kinetic measurements of unfolding and refolding reactions of small model proteins are aimed at the detection and characterization of partially folded intermediates between the unfolded protein and the native state and at the characterization of the rate-limiting steps in the folding process. Fast electron transfer reactions are used to probe the dynamics of the earliest steps in protein folding and of fast conformational transitions. Different experimental techniques are applied that allow us to study conformational dynamics from the femtoseconds to the hours time range.

Structure and Dynamics of Unfolded Proteins

During folding a protein has to form interactions between specific parts of the polypeptide chain. Intrachain diffusion can thus be regarded as the elementary step in the folding process, which determines the maximum rate at which a folding polypeptide chain can explore conformational space. We have developed a model system to directly measure contact formation between two defined points on a polypeptide chain using triplet-triplet energy transfer (TTET).



Schematic representation of TTET experiments from a triplet donor to a triplet acceptor group to measure intrachain diffusion in polypeptide chains.

TTET experiments on formation of short loop of the size of 2 to 10 amino acids had revealed major TTET processes in the dead-time of our standard nanosecond laserflash setup. Based on the fast photophysics of the reactions involved in TTET (Heinz et al., 2006), measurements of loop formation reactions on the sub ns time scale were possible and gave information on the origin of the previously unresolved subnanoscond process. In collaboration with the group of Prof. Wolfgang Zinth (LMU München) we

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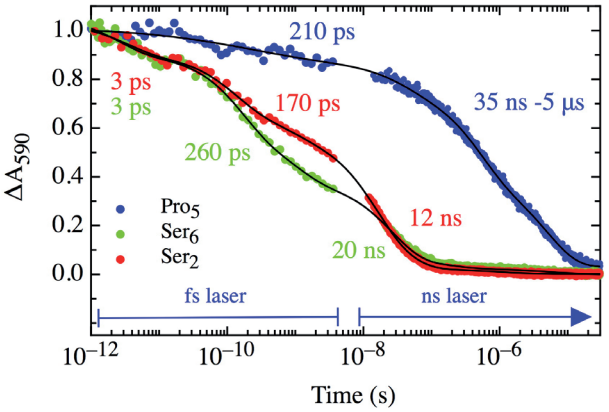
combined results from fs-induced TTET experiments performed in Munich with ns-induced experiments performed in Basel. This allowed us to study loop formation reactions in unfolded polypeptide chains over several orders of magnitude in time from picoseconds to microseconds. The results revealed processes on different time scales indicating motions on different hierarchical levels of the free energy surface. A minor (<10 %) very fast reaction with a time constant of about 3 ps indicates equilibrium conformations with donor and acceptor in contact at the time of the laserflash. Complex kinetics of loop formation were observed on the 50-500 ps time scale which indicate motions within a local well on the energy landscape. Conformations within this well can form loops by undergoing local motions without having to cross major barriers. Exponential kinetics observed on the 10-100 ns time scale are due to diffusional processes involving large scale motions which allow the polypeptide chain to explore the complete conformational space. These results indicate that the free energy landscape for unfolded polypeptide chains and for native proteins have similar properties. The presence of local energy minima reduces the conformational space and accelerates the conformational search for energetically favorable local intrachain contacts. The dynamics of loop formation on different time scales should lead to kinetic heterogeneity in very fast folding processes like formation of α-helices and β-hairpins or acquisition of the three dimensional structure for fast folding proteins (Fierz *et al*., 2006).

To compare the results from TTET on the kinetics of loop formation in unfolded model polypeptide chains with structural properties of the chains we performed time-resolved fluorescence resonance energy transfer (FRET) measurements to gain information on chain dimensions and internal dynamics. Using a novel approach based on global analysis of data obtained from two different donor-acceptor pairs allowed the simultaneous determination of distance distribution functions and diffusion constants between the chromophores. Results on a polypeptide chain consisting of 16 glycine-serine repeats between the FRET chromophores revealed a significant increase in the average end-to-end distance between 0 M and 8 M GdmCl. The increase in chain dimensions is accompanied by an increase in the end-to-end diffusion constant in the presence of denaturant. This suggests that intrachain interactions in water exist even in very flexible chains lacking hydrophobic groups, which indicates intramolecular hydrogen bond formation. The interactions are broken upon denaturant binding, which leads to increased



Dynamics of intramolecular loop formation on the time scale from ps to microseconds for different peptide loops. The sequences are indicated in the legend.

chain flexibility and longer average end-to-end distances. These results imply that rapid collapse of polypeptide chains during refolding of denaturant-unfolded proteins is an intrinsic property of polypeptide chains and can, at least in part, be ascribed to non-specific intramolecular hydrogen bonding. Despite decreased intrachain diffusion constants the conformational search is accelerated in the collapsed state due to shorter diffusion distances. We further used the experimentally determined distance distribution functions and diffusion constants in combination with theoretical models for the kinetics of loop formation which describes rate constants for loop formation as a function of end-to-end diffusion constants, reactive boundary and end-to-end distance distributions. The calculated rate conatnts for loop formation were able to quantitatively reproduce the rate constants for end-to-end loop formation determined by TTET in the same polypeptide chains. This demonstrated the consistency of the data obtained from FRET experiments, which measure distance distributions and local diffusion constants and data from TTET experiments, which directly measure loop formation (Möglich *et al*., 2006).

Folding Mechanism of Small Single Domain Proteins

Several small proteins were shown to fold very fast (typically on the millisecond time scale) and without detectable intermediates. Our group uses several single domain proteins including the DNA-binding domain of brinker, Hsp15, parvalbumin and tendamistat as model systems to study folding and stability of fast folding proteins. To obtain insight into the barriers for protein folding we applied various kinetic concepts

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from physical organic chemistry to protein folding reactions. Analysis of folding data from our lab and of data reported in literature on other proteins allowed us to obtain a general picture of the properties of free energy landscapes for protein folding reactions. The results indicate that folding transition states are narrow regions on the free energy landscape which are robust against perturbations like mutations or changes in solvent conditions. Apparent transition state movements frequently observed in mutational studies were shown to be due to ground state effects, most commonly caused by structural changes in the unfolded state. We could show that the sensitivity in the detection of transition state movements can be improved by applying multiple perturbations. This allowed a more detailed characterization of the shape of the free energy barriers for protein folding reactions (Schätzle & Kiefhaber, 2006).

Folding and Assembly of Oligomeric Proteins

Model proteins for studies on the mechanism of protein folding are usually performed on monomeric proteins. We are currently studying two model proteins to gain insight into the molecular mechanism of folding and assembly of trimeric proteins. In the first approach we study folding and assembly of the C-terminal domain from the trimeric phage T4 protein fibritin. The only known function of this small domain (3x27 amino acids) is the promotion of rapid folding and association of fibritin. Kinetic studies of the folding and assembly pathway revealed a sequence of very fast first order folding and second order association reactions, in accordance with an evolutionary optimized assembly system. Results from equilibrium and kinetic experiments in combination with amino acid replacements at specific positions allowed us to gain insight into the importance of different parts of the protein for the assembly process. The experiments revealed that rapid formation (< 1 ms) of a monomeric intermediate with native-like topology is crucial for rapid protein-protein interactions during the association steps.

In a second approach we study the folding mechanism of collagen. Collagen consists of repetitive Gly-Xaa-Yaa tripeptide units with proline and hydroxyproline frequently found in the Xaa and Yaa position, respectively. This sequence motif allows the formation of a highly regular triple helix that is stabilized by steric (entropic) restrictions in the constituent polyproline-II-helices and backbone hydrogen bonds between the three strands. Concentration-dependent association reactions and slow prolyl isomerization steps have been identified as major rate-limiting processes during collagen folding. To gain information on the dynamics of triple helix formation in the absence of these slow reactions we performed stopped-flow double jump experiments on cross-linked fragments derived from human type III collagen. This allowed us to measure concentration-independent folding kinetics starting from unfolded chains with all peptide bonds in the trans conformation. The results show that triple helix formation occurs with a rate constant of 113±20 s^{-1} at 3.7 °C and is virtually independent of temperature indicating a purely entropic barrier. Comparison of the effect of GdmCl on folding kinetics and stability reveals that the rate-limiting step is represented by bringing 10 consecutive tripeptide units (3.3 per strand) into a triple-helical conformation. The following addition of tripeptide units occurs on a much faster time scale and can not be observed experimentally. These results support an entropy-controlled zipper-like nucleation/growth mechanism for collagen triple helix formation (Bachmann *et al*., 2005).

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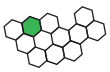

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Conformational dynamics of ATP-driven molecular machines

We study the ATP-driven dynamics of helicases and topoisomerases. These enzyme classes convert the energy of ATP hydrolysis into structural changes of their nucleic acid substrate. Helicases couple the binding and hydrolysis of ATP to unwinding of DNA or RNA duplexes, while topoisomerases introduce supercoils into DNA. In both cases, large conformational changes during the catalytic cycle have been inferred from biochemical and structural data. However, the nature and amplitude of these conformational changes and their role during enzyme activity remain elusive.

RNA helicases

An RNA helicase core domain consists of two α/β domains that are connected by a flexible linker. This core region comprises all conserved helicase motifs required for ATP-dependent RNA unwinding. Additional flanking sequences may mediate interactions with other proteins or confer substrate specificity.

We have determined the crystal structure of the N-terminal helicase subdomain of the RNA helicase Hera from *T. thermophilus* in complex with AMP. This domain contains all motifs involved in nucleotide binding, namely the Q-motif, the Walker A motif (P-loop), the DEAD box, and the SAT motif which has been implicated in the coordination of ATP hydrolysis and RNA unwinding. Upon binding of AMP, the P-loop adopts a partially collapsed or half-open conformation that is still connected to the DEAD box motif, and the DEAD box in turn is linked to the SAT motif via hydrogen bonds. This network of interactions communicates changes in the P-loop conformation to distant parts of the helicase. The affinity of AMP is comparable to that of ADP and ATP, substantiating that the binding energy from additional phosphate moieties is directly converted into conformational changes of the entire helicase. We are currently refining the structure of the C-terminal domains of Hera.

From the observation that the two helicase subdomains have very different relative orientations in various helicase structures, it has been proposed that nucleotide binding to the inter-domain cleft induces a closure of this cleft that is essential for RNA unwinding. To understand the role of conformational changes for ATP-dependent RNA unwinding by helicases, we have analyzed the conformational dynamics of the RNA helicase YxiN from *B. subtilis* in single molecule FRET experiments (Figure 1). In the absence of nucleotide or RNA, the helicase exists in a conformation consistent with an open inter-domain

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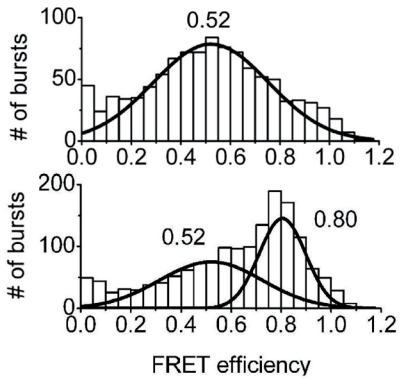

Fig. 1: The pre-hydrolysis state of YxiN is a closed conformation. *Single molecule FRET histograms for donor-acceptor labeled YxiN helicase in the absence of nucleotides (upper panel) and in the presence of the non-hydrolyzable ATP analog ADPNP and RNA substrate (lower panel). Cooperative binding of ATP and RNA induces the formation of a high-FRET species, representing the compact, catalytically active conformation of the helicase. The numbers refer to the mean FRET efficiencies of each species.*

cleft. ATP or RNA binding does not change the global helicase conformation. In the presence of ADPNP and RNA (pre-hydrolysis state), a species with high FRET efficiency becomes populated, consistent with a closure of the inter-domain cleft. In contrast, the helicase adopts an open conformation in the ADP- and RNA-bound state (post-hydrolysis state). Switching events between the open and closed conformations can be observed in real-time.

Comparative single molecule FRET studies of ATP-dependent conformational changes in different helicases will reveal general features of helicase mechanisms and specific features of particular helicases.

To facilitate site-specific introduction of donor and acceptor fluorophores for FRET experiments, we have produced the N-terminal and the C-terminal domains of YxiN separately as intein fusions, and have reconstituted a functional helicase with correct inter-domain communication by expressed protein ligation. By separate labeling of one construct with the donor, the other one with the acceptor, and subsequent ligation, this technique allows for controlled incorporation of fluorescent dyes.

DNA topoisomerases

DNA gyrase is the only topoisomerase that can introduce negative supercoils into DNA at the expense of ATP hydrolysis. Gyrases are A_2B_2 heterotetramers (Figure 2). The GyrA subunit mediates DNA binding and harbors the catalytic tyrosine for transesterification reactions, and the GyrB subunits contain the ATPase sites. While GyrB is a monomer in the absence of nucleotide, its ATP-induced dimerization provides a nucleotide-regulated gate (N-gate). In a current model for the mechanism of DNA supercoiling by gyrases, the so-called two-gate mechanism, a first DNA segment enters through the N-gate, and is cleaved by the active site tyrosines. Subsequently, a second DNA segment enters through the N-gate, is passed through the gap of the cleaved segment (DNA-gate), and exits the gyrase *via* the C-gate (Figure 2). Re-ligation of the first DNA segment ends one catalytic cycle, and ATP hydrolysis allows for re-entering the subsequent functional cycle.

A regulated and coordinated opening and closing of the N-gate, the DNA-gate, and the C-gate has been proposed. Using donor-acceptor labeled gyrase, we investigated the conformational changes of the C- and the DNA-gate in single molecule FRET experiments. The C-gate remains closed during the observation time in the confocal microscope (ca. 10 ms), and there is no significant equilibrium population of gyrase with an open C-gate. Single molecule FRET experiments with surface-immobilized DNA gyrase using a total internal reflection microscope will allow us to increase the observation time, to detect gate

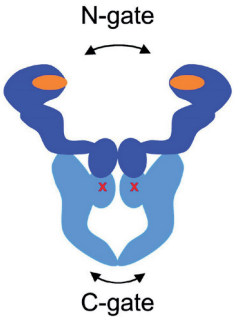

Fig. 2: Putative DNA gyrase mechanism: The two-gate model. *The active gyrase consists of two GyrB subunits (dark blue) and two GyrA subunits (light blue). The ATP binding sites in GyrB are depicted in orange, the active site tyrosines are marked by red crosses.*
According to the two-gate model for negative supercoiling, one dsDNA segment enters through the N-gate and forms a phosphoester intermediate with the tyrosine side-chains. The second dsDNA segment also enters through the N-gate and becomes trapped when ATP binding closes the gate. It passes through the gap in the first DNA segment and eventually leaves the enzyme through the C-gate. The first DNA segment is re-ligated and released.

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opening, and to obtain kinetic information of its opening and closing. Similar experiments addressing the dynamics of the DNA-gate are currently underway.

In addition, we have characterized the *B. subtilis* DNA gyrase with respect to nucleotide binding and hydrolysis, both in the presence and in the absence of DNA. Comparison with the *E. coli* DNA gyrase provides evidence that the basic mechanistic features are conserved among DNA gyrases, but the kinetics of individual steps can vary dramatically even between closely related enzymes. This suggests that each topoisomerase represents a different solution to the complex reaction sequence in DNA supercoiling.

Reverse gyrase is a topoisomerase unique to hyperthermophilic organisms that is capable of positive DNA supercoiling at the expense of ATP hydrolysis. The monomeric enzyme consists of a helicase-like domain fused to a topoisomerase I domain (Figure 3). As a type I topoisomerase, reverse gyrase cleaves only one strand of its double stranded DNA substrate.

Biochemical characterization of *T. maritima* reverse gyrase in the absence and presence of DNA revealed that the enzyme is a DNA-stimulated ATPase already at temperatures that are too low for the supercoiling reaction, indicating that the enzyme is not "frozen" at these temperatures. ADP and the non-hydrolyzable ATP analog ADPNP allow for relaxation of negative supercoils, but ATP hydrolysis is required for the introduction of positive supercoils. Interestingly, ATPγS is bound and hydrolyzed by reverse gyrase at 75°C, and its hydrolysis is accelerated in the presence of DNA. Most importantly, ATPγS promotes positive supercoiling by reverse gyrase with a similar velocity and to a similar extent as ATP.

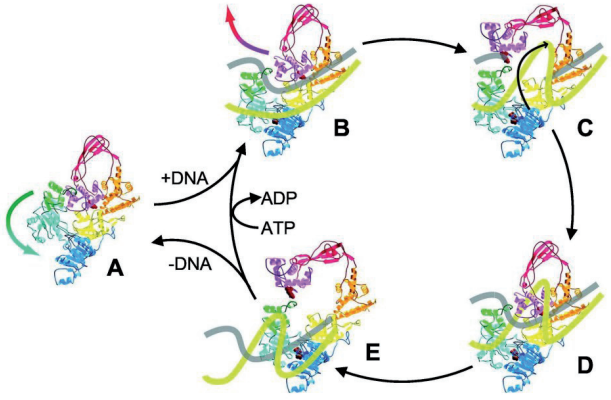

Fig. 3: Proposed catalytic cycle of reverse gyrase
Reverse gyrase structure (A); Binding of double-stranded DNA (B); cleavage of one strand (C), which remains covalently bound to the active site tyrosine via a phosphoester, and strand passage; religation (D); and release via latch opening (E). Large movements of reverse gyrase are required for binding and release of DNA. Modified after Rodriguez & Stock (2002), EMBO Journal 21, 418-426

Large conformational changes in the catalytic cycle of reverse gyrase have been proposed (Figure 3). Future single molecule experiments will provide insight into conformational changes during positive supercoiling.

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The elastic titin filament

The striated muscle of vertebrates undergoes a constant remodelling of its architecture, composition, enzymatic apparatus and innervation in adaptation to mechanical load. Such tissue plasticity is thought to be related to the molecular mechanisms underlying the passive elasticity of the sarcomere. At physiological forces, most of the elastic response of the sarcomere is generated by titin, an intrasarcomeric filamentous protein of >2 um length that consists of serially-linked Ig and FnIII domains (~330 modules) and a single catalytic domain, a Ser/Thr kinase, located near its C-terminus. The spring function of titin resides in its I-band fraction that stretches and recoils during muscle function. Far from being a purely mechanical system, titin is functionalized through the recruitment of active proteins (metabolic enzymes, stress-response factors, transcription factors and signalling modules) to its surface. It is believed that several of the titin-associated proteins can act as mechanically-sensitive transducers that, triggered by force-induced changes, regulate gene expression profiles in the sarcomere. Thereby controlling the trophic state of muscle and coupling signalling to mechanics. Our research aims at deciphering how the titin filament and its associated proteins allow the sarcomere to generate, sense and respond to mechanical tension.

The molecular basis of titin elasticity is currently unknown. The response to stretch of I-band components, particularly poly-Ig arrays, has been extensively studied using nanotools and molecular simulations. These methodologies finger-print unfolding phenotypes defined by the secondary structure elements of component domains (i.e. analyze events at the module level) but do not report on the behaviour of the chain. Since domain unfolding is not the physiological source of titin elasticity, elucidating the structure and dynamics of the interdomain arrangements in the filament is essential to establish its mechanistic principles. In our recent work, we have elucidated the conformational dynamics of poly-Igs from titin at atomic detail and proposed a structure-based mechanistic model of chain elasticity. Based on *i)* a hybrid analysis of the Ig-doublet Z1Z2 using X-ray crystallography, SAXS, NMR relaxation data and residual dipolar couplings (Marino *et al*., 2006); *ii)* an investigation of Z1Z2 energetics using molecular simulations (Lee *et al*., 2007); *iii)* the crystal and SAXS structure of the 6-Ig fragment I65-I70 from the elastic I-band of titin (Figure 1) (von Castelmur *et al*., 2007); and *iv)* an EM study of a recombinant 19-Ig fragment (von Castelmur *et al*., 2007); we concluded that titin Ig-tandems are adaptable modular systems with restricted dynamics which, being connected

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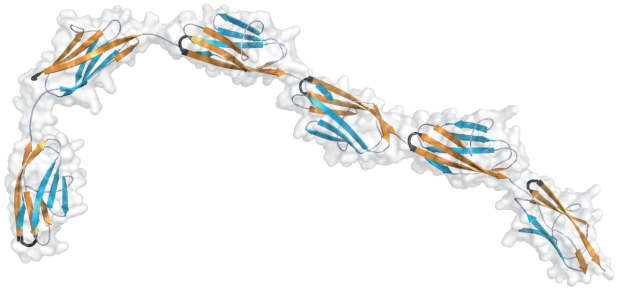

Figure 1: Crystal structure of I65-I70
β-sheets are color coded to emphasize the regularity in domain torsions.

through conserved Ig-Ig transition motifs, lead to the organization of the chain into finely-structured super-motifs. In these, conformationally stiff segments interspersed with pliant hinges form a regular pattern of dynamic units inducing segmental flexibility in the chain. Pliant hinges support molecular shape rearrangements that dominate chain behavior at moderate stretch, while stiffer segments predictably oppose the high stretch forces that develop upon full chain extension, acting as energy barrier to prevent Ig unfolding and triggering, instead, the unraveling of flanking springs formed by PEVK sequences. This modulates the interplay of I-band springs. Titin elasticity should then be expressed in terms of a discreet formalism of freely jointed rigid-segments (FJSC) and not through continuous models such as the worm-like chain (WLC) commonly applied to this system that assumes a smoothly-flexible chain of homogeneous composition. The FJSC mechanistic model incorporates now the primary chemical information of the chain and allows calculating its physical spring properties, offering great promise for the future rationalization and modeling of the stretch-response phenotype of skeletal myofibrils.

In addition to its mechanical function, the recruitment of sarcomeric proteins by titin plays a major role in muscle regulation. Near its C-terminus and directly preceding a kinase domain, titin contains a conserved pattern of Ig and FnIII modules (Ig168-Ig179-Fn170) that recruits the E3 ubiquitin-ligase MuRF-1 to the filament. MuRF-1 is a muscle-specific catabolic factor that mediates myofibril turnover. It has been recently proposed as a therapeutic target for the control of muscle wasting associated to numerous diseases and physiological states such as aging, microgravity, immobilization, cancer, diabetes and AIDS. The function of MuRF-1 appears modulated by its interaction with titin. We have elucidated the crystal structure of Ig168-Ig179-Fn170 and characterized its interaction with MuRF-1 identifying the molecular determinants of the binding (Mrosek et al., 2007). Ig168-Ig179-Fn170 shows an extended, rigid architecture, characterized by a surface ridge that spans its full length and a distinct loop protrusion in its middle point (Figure 2). In MuRF-1, a C-terminal helical domain is sufficient to bind titin with high affinity. This helical region predictably docks into the surface groove of Ig168-Ig179-Fn170, where the loop protrusion is a key mediator of MuRF-1 recognition. Our findings indicate that also this region of titin could serve as a target to attempt therapeutic inhibition of MuRF-1-mediated muscle turnover.

Finally, building on our knowledge of titin structure and interactions, we are exploring new design avenues for the nanofabrication of functionalized, fibrous biomaterials for use in biomedicine and as analytic tools in cell biology.

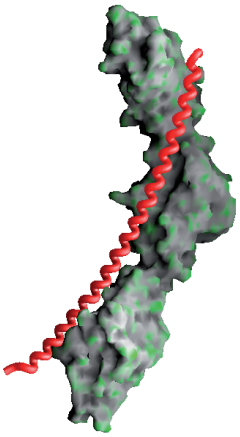

Figure 2: Crystal structure of A168-A170
Surface representation emphasizing the ridge in the concave side of the molecule. Modelling of a docked α-helical peptide in coiled-coil conformation, representative of the C-terminal domain of MuRF-1.

Evolutionary events in the tryptophan biosynthesis pathway

Enzyme components of the tryptophan biosynthesis pathway constitute one of the richest model systems to explore the evolutionary principles of macromolecular recognition, channelling, allosterism and functional diversification in protein networks.

One of the enzymes under study in our laboratory is TrpD, anthranilate phosphoribosyltransferase (AnPRT), which links this pathway to aspects of nucleotide metabolism. The metabolic synthesis and

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degradation of essential nucleotide compounds is primarily carried out by phosphoribosyltransferases (PRT) and nucleoside phosphorylases (NP), respectively. Despite the resemblance of their reactions, five unrelated families of PRTs and NPs exist, where AnPRT constitutes the only evolutionary link between synthesis and salvage processes. Recently, we have characterized the active site of dimeric AnPRT from *Sulfolobus solfataricus* by elucidating crystal structures of the wild-type enzyme complexed to its two natural substrates: anthranilate and PRPP/Mg^{2+}, revealing the active site at full occupancy (Marino *et al*., 2006) (Figure 3). The anthranilate and PRPP/Mg^{2+} substrates bind into two different domains within each protomer and are brought together during catalysis by rotational domain motions that we have revealed by small angle X-ray scattering data. The motions, however, do not result in cooperativity and protomers within the biological dimer act as independent catalytic units (Schwab *et al*., 2007). Steady-state kinetics of mutated AnPRT variants (Marino *et al*., 2006) address the role of active site residues in binding and catalysis. Results allow the comparative analysis of PRT and pyrimidine NP families and expose structural motifs that constitute the common basis for nucleotide/nucleoside recognition by these evolutionarily unrelated enzymes.

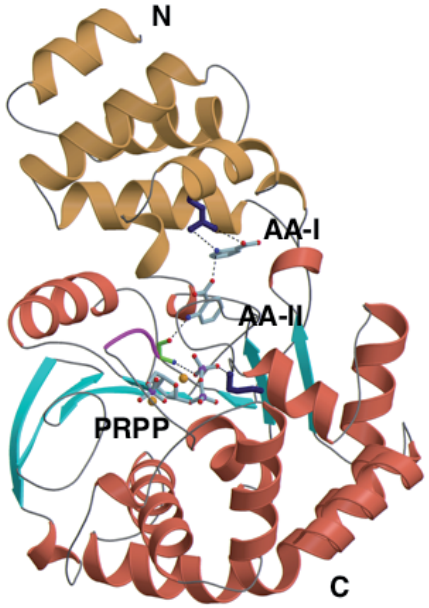

Figure 3: Crystal structure of AnPRT in complex with its substrates anthranilate and PRPP/Mg2+. The active site is shown at full occupancy. R164 and K106, mediating conserved interactions across AnPRTs and PyNPs, are displayed in dark blue.

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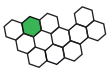

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Crystallography of bacterial membrane transport and signaling proteins

We are studying the structural determinants for solute translocation across the outer and the inner bacterial membrane. Our second focus is on proteins that are involved in signal transduction utilizing the cyclic dinucleotide c-di-GMP, a recently discovered ubiquitous second messenger in prokaryotes.

Sugar transporters of the bacterial phosphoryl-transfer-system

The PEP-dependent carbohydrate:phosphotransferase system (PTS) couples solute translocation across the inner membrane to solute phosphorylation. The PTS is ubiquitous in bacteria, but not found in eukaryotes rendering it an attractive target for novel antibiotics. The PTS consists of general proteins (enzyme I and HPr) and sugar-specific components (enzymes II). Latter are comprised of several structural domains or subunits: IIA and IIB are hydrophilic cytoplasmic components and catalyze the phosphorelay, whereas IIC (IID) is integrated in the plasma membrane and catalyzes translocation. We have been studying the mannose specific PTS and have solved the structures of the IIA and IIB domains. The transition state for phosphoryl transfer between IIA and IIB has been modeled based on the individual X-ray structures. The model of the complex shows surface and charge complementarity at the interface. The penta-coordinated phosphoryl group appears to be stabilized by the partial dipole charges of two helices, one from each protein.

Presently, the major effort of our research is the structure determination of sucrose permease IIBC from *Salmonella typhimurium*. IIBC is composed of two domains: the membrane-spanning IIC domain that is covalently linked to a cytosolic IIB domain. This protein was chosen, because its IIB domain may facilitate crystal formation by providing additional hydrophilic surface. Active IIBC has been successfully expressed in *E. coli* and purified. The homogeneity of the sample was verified by ultracentrifugation and electron microscopy. Extensive screening of crystallization conditions has been undertaken, but crystals of suitable quality for structure determination have not yet been obtained. In parallel, we are attempting to crystallize IIBC in complex with antibody Fab fragment. Respective monoclonal antibodies have been produced and selected for their ability to bind conformational epitopes. Crystallization assays of Fab-IIBC$_{Suc}$ complexes are underway.

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Porins

Porins are integral membrane proteins from the outer membrane of Gram-negative bacteria. They allow the uptake of nutrients by passive diffusion through an intrinsic pore that extends along the axis of the transmembrane β-barrel structure. With the structure of OmpC, the group has now determined the structures of all major *E. coli* porins. More work has to be done, however, to understand in which molecular properties OmpC excels over OmpF under certain physiological conditions.

Recently, several new porins displaying properties different to those of the classical and structurally well characterized porin families have been reported. We are studying KdgM, a major outer membrane protein of the plant pathogen Erwinia chrysanthemi, that is thought to act as an oligogalacturonate specific porin used by the bacteria during the course of plant infection. In collaboration with G. Condemine (Villeurbanne, France), KdgM, as well as homologues from *Escherichia coli* (NanC) and *Yersinia pestis* (KdgN), have been overexpressed and purified. Recently, we were able to determine the structure of NanC which is probably specific for N-acetyl neuraminic acid. The 2.0 Å crystal structure of this outer membrane protein displays a 12-stranded β-barrel with a highly charged interior. Currently, we are investigating the structural basis for specific substrate translocation.

Diguanylate cyclases and regulation of c-di-GMP synthesis

Recent discoveries suggest that a novel second messenger, c-di-GMP, is extensively used by bacteria to control multicellular behavior, such as biofilm formation. Condensation of two GTP to the dinucleotide is catalyzed by the widely distributed diguanylate cyclase domain that occurs in various combinations with sensory and/or regulatory modules. In collaboration with the group of Urs Jenal (Biozentrum) we are studying, amongst others, PleD, an essential part of the signaling pathway regulating the developmental cycle of *Caulobacter crescentus*. The response regulator is composed of two (CheY like) input domains and a highly regulated diguanylate cyclase. Crystal structures of native and activated, i.e. beryllofluoride modified, PleD allowed us to propose and corroborate a mechanistic model of PleD activation and non-competitive product inhibition. Accordingly, phosphorylation of the enzyme at the receiver domain promotes dimerization, the prerequisite for the efficient encounter of two substrate loaded active sites. Allosteric inhibition, on the other hand, occurs via the immobilization of the catalytic domains by c-di-GMP induced cross-linking to the stem (3) or, alternatively, to the second catalytic domain in the dimer (4). This probably sets an upper limit for the cellular c-di-GMP concentration in the micromolar range.

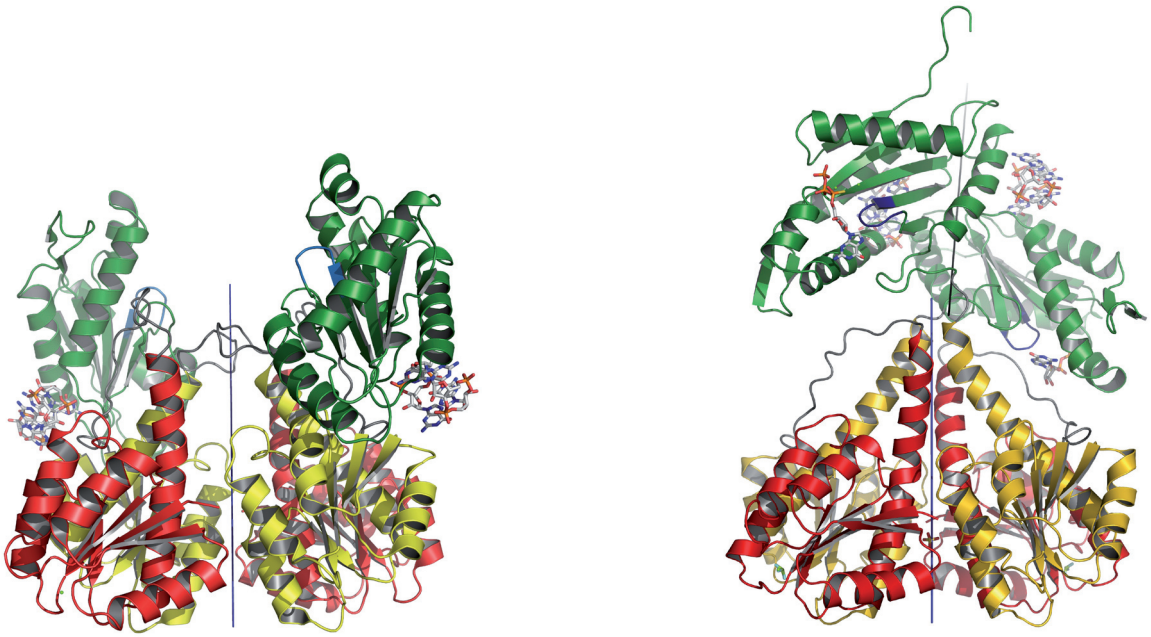
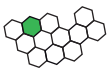


Figure: PleD in its native (left) and activated (right) state. Activation by beryllofluoride at the receiver domain (red) results in tightening of the dimeric stem (red and yellow domains). Also seen is a different arrangement of the DGC domains (green) due to distinct domain cross-linking by c-di-GMP.



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To contribute further to our knowledge about the regulation of c-diGMP levels in bacterial cells we also have started to investigate other diguanylate cyclases and c-di-GMP specific phosphodiesterases.

Effector proteins of the type IV secretion system

Type IV secretion systems (T4SS) are utilized by many bacterial pathogens for the delivery of virulence proteins or protein-DNA complexes into their eukaryotic target cells. We are working on a class of effector proteins that are composed of a Fic and a BID domain responsible for pathogenic action in the host cell and for translocation, respectively. In collaboration with the Dehio group (Biozentrum), we have revealed the structure the Fic domain of BepA from *Bartonella henselae*. Investigations into the function of these modules are underway.

Plant and insect allergens and allergen – antibody complexes

Type I allergy is a hypersensitivity disease affecting more than 25% of the population. It is characterized by an increased production of IgE antibodies against otherwise harmless antigens, i.e. allergens. Specific immunotherapy (SIT) with natural extracts is an effective treatment of type I allergy, but a great disadvantage is the danger of associated IgE-mediated anaphylactic side effects. The objective of this research is to determine the structural and immunological characteristics of major allergenic proteins from insect and plants. The crystal structure of allergens defines the surface exposed residues which are most likely to interact with antibodies, whereas the crystal structure of allergens in complex with specific antibody (IgE Fabs or IgG Fabs which compete with IgE) reveals the detailed molecular interactions in the allergen-antibody interface.

Towards this objective, we have determined the crystal structure of a complex between the major allergen from bee venom (Hyal) and an IgG1 Fab fragment. The Fab fragment recognizes a conspicuous protruding helix-turn-helix substructure of the antigen. Recently, the structure of major timothy grass pollen allergen Phl p 2 in complex with a specific human IgE Fab fragment. Here, the antibody recognizes the flat surface of the β-sheet. The IgE epitope structures may allow for a rational design of hypoallergens with reduced IgE binding capacity, but retained T-cell recognition (hypoallergens) for allergen specific immunotherapy.

In addition, the X-ray structures of two major allergens have been determined, Dau c 1 from carrot and Sol i 3 from the fire ant. The structure of Dau c 1 is almost identical to that of the major celery allergens (Api g 1) and contains the same set of epitopes which may explain the observed cross-reactive with the homologous major birch pollen allergen Bet v 1. The structure of Sol i 3 from the fire ant is arranged to an α-β-α sandwich. The identified surface exposed residues may now be subjected to site directed mutagenesis aimed at producing hypoallergens.

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Actin in the nucleus: what forms for what functions

In recent years, a number of cellular processes have been connected with nuclear actin including mechanical support of a nuclear matrix, stabilization of the nuclear lamina, chromatin remodeling, complex formation with hnRNP particles, and activity of all three types of RNA polymerase, but how and in what form actin participates in these events is largely unknown. The variety of nuclear functions could be rooted in the plasticity of the actin molecule and/or in the assembly of 'unconventional' forms of actin. Consistent with this hypothesis are findings in which antibodies reveal different staining patterns of nuclear and cytoplasmic actin within a cell. For example, the monoclonal antibody (mAb) 2G2 (Gonsior *et al.*, 1999), which was raised against a profilin–actin complex, recognizes actin particles in the nucleus, the submembraneous actin web and also in filopodial extensions of cultured cells. The 2G2 epitope was identified in a region of the actin monomer that is not exposed on the surface of subunits incorporated in F-actin filaments but is presented when actin is complexed with profilin. This finding shows that actin-binding proteins (ABPs), through their interaction with actin, can 'mold' different forms of actin at different cellular sites. Therefore, it is essential to identify the target proteins of actin in specific cellular compartments. We have previously reported that 1C7, a mAb raised against a chemically crosslinked dimer of actin ('lower dimer', LD) also stains actin in the nucleus, but the pattern is quite different from that seen with 2G2. A straightforward interpretation of the results is that these antibodies recognize two different forms of non-filamentous actin.

Using peptide nanoparticles as a platform for the immunogenic display of poor antigenic determinants

Antibodies that discriminate different forms of actin by their surface exposed epitopes, are important tools to examine the patterning of actin. However, because actin is an evolutionary highly conserved molecule, it is a poor antigen and thus, extremely few antibodies are available that specifically recognize distinct forms of actin.

Display of peptides or proteins in an ordered, repetitive array, such as on the surface of a virus-like particle, is known to induce an enhanced immune response relative to vaccination with the „free" protein antigen. To achieve a repetitive and ordered antigenic display of actin-related epitopes, we grafted them onto a peptide nanoparticle (NP) platform that was computationally designed to form regular icosahedrons (Raman *et al.*, 2006). Recombinantly

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expressed polypeptides that self-assembled into spherical NPs were used to generate monoclonal and polyclonal antibodies. Several mAbs were established that recognize the actin 'hydrophobic loop', a structural feature of the actin molecule, both, as an epitope on the surface of NPs, as well as in a cellular environment. Our findings demonstrated that peptide nanoparticles are a suitable display platform to elicit an immune response against poor antigenic determinants in different hosts. To this end, we have employed this experimental system to generate a number of antibodies that are valuable tools for analyzing different forms of actin in different cellular compartments. In addition, we have taken advantage of the repetitive display of epitopes on NPs to establish mAbs that specifically react with the actin binding protein profilin I and mAbs that detect an N-terminal fragment of the human raver1. Both of these antibody tools will be useful in studying the interactions of actin with target proteins in the nucleus.

Unconventional forms of actin

The 'lower dimer' (LD) is a transient actin species where the subunits are arranged in an antiparallel orientation and establish intersubunit contacts that do not occur in F-actin filaments at steady state. Because of its transient nature, LD has so far escaped detection *in vivo*. Transmission electron microscopy of *in vitro* copolymerization experiments showed that covalently crosslinked LD could be incorporated into actin filaments and thereby significantly altered the morphology of conventional F-actin filaments. Antibodies that specifically react with LD would be key tools to further investigate the role of the LD in actin patterning *in vitro* and *in vivo*. Because NP platforms were shown to turn poor antigens into powerful immunogens, we have taken a corresponding approach to obtain mAbs that specifically detect the LD conformation of actin. Based on structural data available, epitopes were designed that are unique to an antiparallel arrangement of actin subunits and recombinantly engineered onto the surface of NPs. Immunization with corresponding NPs yielded a number of mAbs that specifically bind to the LD-related epitopes on NPs. Preliminary immunofluorescence studies suggest that the epitope is also present in cells. Current efforts are aimed at characterizing these mAbs with respect to their specificity for the LD conformation.

Interaction of actin with raver1

Raver1 is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family and has been shown to interact with the ABPs (meta)vinculin and α-actinin, besides binding to the poly-pyrimidine-tract binding protein (PTB). Consistent with the diverse nature of its binding partners, raver1 has been found to reside not only in the nucleus, but also at specific cytoplasmic locations. Little is known about the functions of this protein, which can shuttle between the nucleus and the cytoplasm. On one hand, its association with PTB suggests a role in alternative splicing events, on the other hand, its translocation to the I-Z-I band during muscle differentiation5 and the interaction with microfilament-associated (meta)vinculin and α-actinin point to a role in linking gene expression to structural functions of the contractile machinery of muscle. Preliminary data from pull-down assays with nuclear extracts and cosedimentation experiments of actin and raver1 suggest that the two proteins interact. Further support for an actin-raver interaction is provided by studies showing binding of actin to other hnRNPs. To further characterize the interaction with actin, we plan to carry out binding experiments with recombinantly expressed raver1 fragments. However, expression of full-length raver1 or the C-terminal fragment in *E. coli* has so far not been succesful. Therefore, we are currently exploring alternative expression systems.

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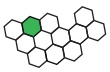

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The Role of ABC Transporters in Membrane Permeation of Drugs

Our group is interested in the passive and active transport of drugs across biological membranes, in particular the blood-brain barrier (BBB). The main focus is on the quantitative understanding of multidrug resistance produced by ABC transporters such as P-glycoprotein (MDR1, ABCB1).

Unraveling membrane-mediated substrate-transporter interactions

Most well-known transporters move either just one substrate or a single class of substrates across the membrane whereby, substrate binding and release occures in the aqueous phase. P-glycoprotein (MDR1, ABCB1) differs by taking care of a broad range of chemically diverse substrates and moving them from the inner to the outer membrane leaflet at the expense of ATP hydrolysis. Substrate binding and most likely also release, thus take place in the lipid phase. Direct measurement of substrate-transporter recognition in the lipid phase has not yet been possible.

In comparison to the aqueous milieu the anisotropic lipid environment changes many physical-chemical parameters. Among others, it partially or totally strips off the hydration shell of the partitioning molecule, induces preferential molecular orientation, stabilizes secondary structural elements such as α-helix and β-sheet, in the case of peptides, and increases the rate of translational diffusion to the protein target. It also enhances electrostatic interactions, including H-bonding interactions, due to its lower dielectric constant.

The binding of a substrate from water to the activating binding region of P-glycoprotein has been shown to occur in two steps, a partitioning step from water to the lipid membrane, characterized by the lipid-water partition coefficient, K_{lw}, and a binding step from the lipid membrane to the transporter, characterized by the transporter-lipid binding constant, K_{tl}. The transporter-water binding constant, K_{tw}, can thus be expressed as product of two individual binding constants K_{lw}, and K_{tl}. We experimentaly determined the binding constants K_{tw} and K_{lw} which allowed for the first time quantitative estimation of the binding affinity of the drug to the transporter (indicated in yellow in Figure 1). To address the most intriguing question as to the nature of the substrate-transporter interactions an educated guess is still required. Based on the observation that all P-glycoprotein substrates carry H-bond acceptor groups (indicated as A in Figure 2) arranged in specific pat-

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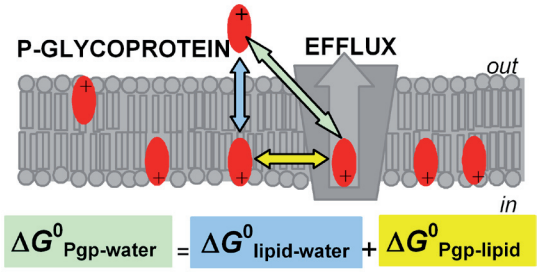

Figure 1: The binding affinity of the drug from water to the transporter ($\Delta G°_{tw}$, green) is the sum of the binding affinity of the drug from water to the lipid membrane ($\Delta G°_{lw}$, blue) and the binding affinity of the drug from the lipid membrane to the transporter ($\Delta G°_{tl}$, yellow). The latter is most likely due to hydrogen bonding interactions.

terns and that the putative transmembrane sequences of P-glycoprotein are rich in H-bond donor groups a modular recognition process based on H-bond formation was proposed (Figure 2) (Gatlik-Landwojtowicz *et al*., 2006 *Biochemistry* 45, 3020-3032).

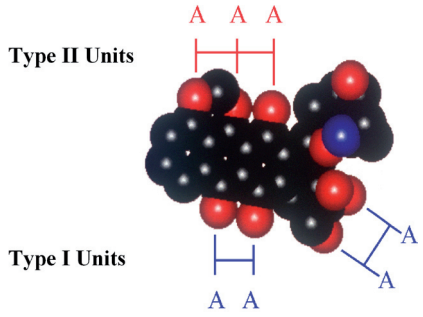

Figure 2: Hydrogen bond acceptors (A) in drugs form different patterns (type I and type II units) which function as binding modules for P-glycoprotein.

P-glycoprotein kinetics measured in plasma membrane vesicles and living cells

P-glycoprotein (MDR1, ABCB1) is an ATP-dependent efflux transporter of a large variety of compounds. To understand P-glycoprotein in more detail, it is important to elucidate its activity in the cellular ensemble as well as in plasma membrane vesicles (under conditions where other ATP dependent proteins are blocked). We measured P-glycoprotein activity in inside-out vesicles formed from plasma membranes of *MDR1*-transfected mouse embryo fibroblasts (NIH-MDR1-G185) for comparison with previous measurements of P-glycoprotein activity in living NIH-MDR1-G185 cells. In plasma membrane vesicles activity was measured by monitoring phosphate release upon ATP hydrolysis and in living cells by monitoring the extracellular acidification rate upon ATP synthesis. P-glycoprotein was stimulated as a function of the concentration with 19 structurally different drugs, including local anesthetics, cyclic peptides, and cytotoxic drugs. The concentrations of half-maximum P-glycoprotein activity, K_1, were identical in inside-out plasma membrane vesicles and in living cells and covered a broad range of concentrations (K_1 ~ (10^{-8} -10^{-3}) M). The influence of the pH, drug association, and vesicle aggregation on the concentration of half-maximum P-glycoprotein activation was investigated. The turnover numbers in plasma membrane vesicles and in living cells were approximately identical if the latter were measured in the presence of pyruvate. However, in the absence of pyruvate they were higher in living cells. The rate of ATP hydrolysis/ATP synthesis decreased exponentially with increasing binding affinity of the drug to the transporter (Figure 3) (Äänismaa and Seelig 2007, *Biochemistry* 46, 3394-3404).

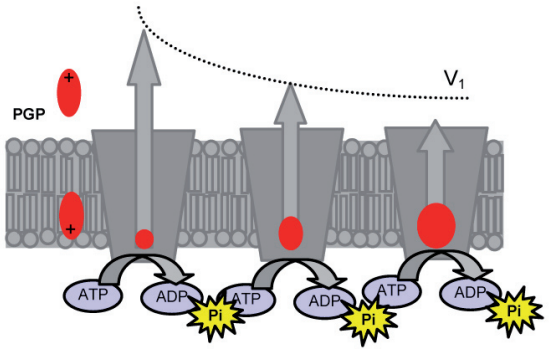

Figure 3: Drugs are shown in red. Drugs with low affinity are indicated as small, and drugs with high affintiy as large circles. The rate of ATP hydrolysis by P-glycoprotein is indicated as grey arrow. The dotted line indicates the exponential decrease of the rate of ATP hydrolysis.

Enhancement of drug absorption by noncharged detergents through membrane and P-glycoprotein binding

Noncharged detergents are used as excipients in drug formulations. Until recently, they were considered as inert compounds, enhancing drug absorption essentially by improving drug solubility. However, many detergents insert into lipid membranes, although to different extents, and change the lateral packing density of membranes at high concentrations. Moreover, they bind to the efflux transporter P-glycoprotein and most likely also to related trans-

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porters and metabolising enzymes with overlapping substrate specificities. If their affinity to P-glycoprotein is higher than that of the coadministered drug they act as modulators or inhibitors of P-glycoprotein and enhance drug absorption. Inhibition of P-glycoprotein and related proteins can, however, cause severe side effects. We reviewed the membrane binding propensity of different noncharged detergents (including poloxamers) and discusses their ability to bind to P-glycoprotein. Literature data on drug uptake enhancement by noncharged detergents, obtained *in vivo* and *in vitro*, were analysed at the molecular level. The analysis provides the tools for an approximate and simple prior estimate of the membrane and P-glycoprotein binding ability of noncharged detergents based on a modular binding approach. (Seelig and Gerebtzoff 2006, *Exp. Opin. Drug Metab. Toxicol*. 5, 733-752).

In silico prediction of blood-brain barrier permeation using the calculated molecular cross-sectional area as main parameter

The cross-sectional area, A_D, of a compound oriented in an amphiphilic gradient such as the air-water or lipid-water interface has been shown to be crucial for membrane partitioning and permeation, respectively (Fischer *et al*., 1998. *J. Membrane Biol*. 165, 201-211). We therefore developed an algorithm that determines the molecular axis of amphiphilicity and the cross-sectional area, A_{Dcalc}, perpendicular to this axis. Starting from the conformational ensemble of each molecule, the three-dimensional conformation selected as the membrane-binding conformation was the one with the smallest cross-sectional area, A_{DcalcM}, and the strongest amphiphilicity. The calculated, A_{DcalcM}, and the measured cross-sectional areas, A_D, correlated linearly (n = 55, slope, m = 1.04, determination coefficient, r^2 = 0.95). The calculated cross-sectional areas, A_{DcalcM}, were then used together with the calculated octanol-water distribution coefficients, Log$D_{7.4}$, of the 55 compounds (with known ability to permeate the blood-brain barrier) to establish a calibration diagram for the prediction of blood-brain barrier permeation. It yielded a limiting cross-sectional area (A_{DcalcM} = 70 Å^2) and an optimal range of octanol-water distribution coefficients (1.4 ≤ Log$D_{7.4}$ < 7.0). The calibration diagram was validated with an independent set of 43 compounds with known ability to permeate the blood-brain barrier, yielding a prediction accuracy of 86%. The incorrectly predicted compounds exhibited Log$D_{7.4}$ values comprised between -0.6 and -1.4, suggesting that the limitation for Log$D_{7.4}$ is less rigorous than the limitation for A_D. An accuracy of 83% has been obtained for a second validation set of 42 compounds which were previously shown to be difficult to predict. The calculated parameters, A_{DcalcM} and Log$D_{7.4}$ thus allow for fast and accurate prediction of blood-brain barrier permeation. Analogous calibration diagrams can be established for other membrane barriers. (Gerebtzoff and Seelig 2006, *J. Chem. Inf. Model* 46, 2638-2650).

The role of size and charge for blood-brain barrier permeation of drugs and fatty acids

The lipid bilayer is the diffusion barrier of biological membranes. Highly protective membrane barriers such as the blood-brain barrier (BBB) are reinforced by ABC transporters including P-glycoprotein (MDR1, ABCB1) and multidrug resistance associated proteins (MRPs, ABCCs). They bind their substrates in the cytosolic lipid bilayer leaflet before they reach the cytosol and flip them to the outer leaflet. Since the large majority of drugs targeted to the CNS are intrinsic substrates of these transporters passive influx (Φ) is counteracted by active efflux (-V). Whether an intrinsic substrate can cross the BBB thus depends on whether passive influx is higher than active efflux. Here we show that passive influx (Φ) can be estimated quantitatively on the basis of Stokesian diffusion, taking into account the pKa and the cross-sectional area of the molecule in its membrane bond conformation, as well as the lateral packing density of the lipid bilayer. Active efflux (-V) by ABC transporters was measured. The calculated net flux that is the sum of passive influx and active efflux is in excellent agreement with experimental results. The approach is exemplified with several drugs and fatty acid analogs, and shows that compounds with small cross-sectional areas (A_D < 70 Å^2) and/or intermediate or low charge exhibit higher passive influx (Φ) than active efflux (V) and therefore cross the BBB

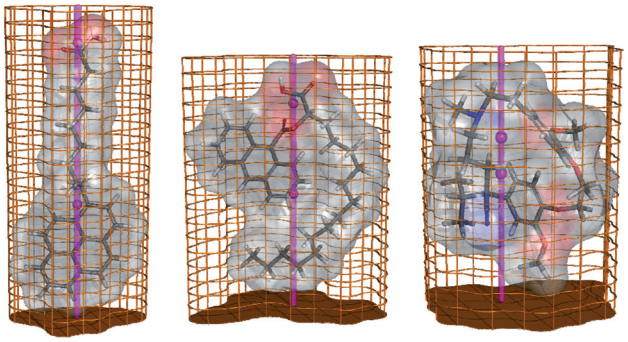
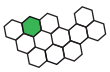


Figure 4: Pyrene nonanoic acid (A_D < 70 Å^2), antroyloxystearic acid, and verapamil (A_D > 70 Å^2). The molecules are shown in their membrane bound, amphiphilic conformation as described in Gerebtzoff and Seelig (2006).



despite being intrinsic substrates, whereas large or highly charged compounds (A_D > 70 Å^2) show higher efflux than influx. They are apparent substrates and cannot cross the BBB. The strict size and charge limitation for BBB permeation results from the synergistic interaction between passive influx and active efflux. (Seelig 2007, *J. Mol. Neurosc.* 33, 32-41).

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Biophysical Techniques in Membrane Research and in *in vivo* Biochemistry

The group of J. Seelig is concerned with mainly two topics: (1) the membrane-induced random-coil-to-β-structure transition as observed for Alzheimer peptide Aβ and (2) the action mechanism of cell penetrating peptides (CPPs) such as TAT-PTD, different polyarginines (R5-R9), and dioleoylmelittin (DOM).

Thermodynamics of the Coil-to-β-Sheet Transition in a Membrane Environment

Biologically important peptides such as the Alzheimer peptide Aβ(1-40) display a reversible random coil-to-β-structure transition at anionic membrane surfaces. In contrast to the well-studied random coil-to-β-helix transition of amphipathic peptides there is a dearth on information on the thermodynamic and kinetic parameters of the random coil-to-β-structure transition. We have analyzed the thermodynamic parameters of the membrane-induced β-structure formation using the model peptide (KIGAKI)3 and 8 analogs in which two adjacent amino acids were substituted by their D-enantiomers. The positions of the d,d pairs were systematically shifted along the three identical segments of the peptide chain. The β-structure content of the peptides was measured in solution and when bound to anionic lipid membranes with circular dichroism spectroscopy. The thermodynamic binding parameters were determined with isothermal titration calorimetry and the binding isotherms were analyzed by combining a surface partition equilibrium with the Gouy-Chapman theory. The thermodynamic parameters were found to be linearly correlated with the extent of β-structure formation. β-structure formation at the membrane surface is characterized by an enthalpy change of ΔH_β = -0.23 kcal/mol per residue, an entropy change of ΔS_β = -0.24 cal/mol K residue and a free energy change of ΔG_β = -0.15 kcal/mol residue. Increase in temperature induces an unfolding of β-structure. The residual free energy of membrane-induced β-structure formation is close to that of membrane-induced α-helix formation. (Meier and Seelig 2007, *J. Mol. Biol*. 369, 277-289; Meier and Seelig, *J. Amer. Chem. Soc*., in press).

Thermodynamic Studies on the Interaction of Antibodies with β-Amyloid Peptide

Antibodies against β-amyloid peptides (Aβs) are considered an important therapeutic opportunity in Alzheimer's disease. Despite the vast interest in Aβ no thermodynamic data on the interaction of antibodies with Aβ is available as yet. In the present study we use isothermal titration calorimetry (ITC) and surface plasmon resonance to provide a quantitative thermodynamic analysis of the interaction

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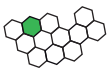

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between soluble monomeric Aβ(1-40) and mouse monoclonal antibodies (mAb). Using four different antibodies directed against the N-terminal, middle, and C-terminal Aβ epitopes we measured the thermodynamic parameters for the binding to Aβ. Each antibody species was found to have 2 independent and equal binding sites for Aβ with binding constants in the range of 10^7 M^{-1} to 10^8 M^{-1}. The binding reaction was essentially enthalpy-driven with a reaction enthalpy of $\Delta H^0_{A\beta}$ ~ -19 kcal/mol to -8 kcal/mol, indicating the formation of tight complexes. The loss in conformational freedom was supported by negative values for the reaction entropy $\Delta S^0_{A\beta}$. We also measured the heat capacity change of the 1 mAb:2Aβ reaction. $\Delta C^0_{p,A\beta}$ was large and negative but could not be explained exclusively by the hydrophobic effect. The free energy of binding was found to be linearly correlated with the size of the epitope. (Brockhaus *et al*. 2007, *J. Phys. Chem. B*, 1238-1242).

Leakage and Lysis of Lipid Membranes Induced by the Lipopeptide Surfactin

Surfactin is a lipopeptide produced by Bacillus subtilis which possesses antimicrobial activity. We have studied the leakage and lysis of POPC vesicles induced by surfactin using calcein fluorescence dequenching, isothermal titration calorimetry and ^{31}P solid state NMR. Membrane leakage starts at a surfactin-to-lipid ratio in the membrane, $R_b \approx 0.05$, and an aqueous surfactin concentration of $C^w_s \approx 2$ μM. The transient, graded nature of leakage and the apparent coupling with surfactin translocation to the inner leaflet of the vesicles, suggests that this low-concentration effect is due to a bilayer-couple mechanism. Different permeabilization behaviour is found at $R_b \approx 0.15$ and attributed to surfactin-rich clusters, which can induce leaks and stabilize them by covering their hydrophobic edges. Membrane lysis or solubilization to micellar structures starts at $R^{sat}_b = 0.22$ and $C^w_s = 9$ μM and is completed at $R^{sol}_m = 0.43$ and $C^w_s = 11$ μM. The membrane–water partition coefficient of surfactin is obtained as $K = 2 \times 10^4$ M^{-1}. These data resolve inconsistencies in the literature and shed light on the variety of effects often referred to as detergent-like effects of antibiotic peptides on membranes. The results are compared with published parameters characterizing the haemolytic and antibacterial activity. (Heerklotz and Seelig 2007, *Eur. Biophys. J*. 36, 305-314).

High Affinity of the Cell-Penetrating Peptide HIV-1 Tat-PTD for DNA

Cell-penetrating peptides (CPPs) are cationic peptides which, when linked to genes, proteins, or nanoparticles, facilitate the transport of these entities across the cell membrane. A fairly large range of chemicals is commercially available but in spite of their potential use for gene transfer, drug delivery, and intracellular imaging the mode of action of CPPs is still mysterious. It has even been argued that the observed transport across the cell membrane is an artifact caused by chemical fixation of the cells, a common preparation method for microscopic observation. We have therefore synthesized a fluorescent derivative of the HIV-1 TAT protein transduction domain (Fg-$CPP^{TAT(PTD)}$) and have observed its uptake into non-fixated living fibroblasts with time-lapse confocal microscopy, eliminating the need for fixation. We find that Fg-$CPP^{TAT(PTD)}$ enters the cytoplasm and nucleus of non-fixated fibroblasts within seconds arguing against the suggested artifact of cell-fixation. With differential interference contrast microscopy we furthermore detect dense aggregates on the cell surface. Several observations suggest that these aggregates consist of Fg-$CPP^{TAT(PTD)}$ bound to membrane-associated heparan sulfate (HS). The aggregates grow in parallel with Fg-$CPP^{TAT(PTD)}$ uptake and are detected only on fibroblasts showing Fg-$CPP^{TAT(PTD)}$ uptake. The aggregation of Fg-$CPP^{TAT(PTD)}$ on the membrane surface bears close resemblance to earlier reports of „capping“ of cell surface molecules combined with a polarized endocytotic flow. Enzymatic removal of extracellular HS reduced both Fg-$CPP^{TAT(PTD)}$ uptake and aggregate formation demonstrating that HS is involved in the uptake mechanism. We have also investigated the effect of non-labeled $CPP^{TAT(PTD)}$ on the metabolism of intact fibroblasts by measuring the extracellular acidification rate (ECAR) of fibro blasts with a cytosensor microphysiometer. Short exposure (<2.5 min) of the cells to CPP concentrations of 500 μM caused a 22% reductionof the ECAR which was however reversible upon superfusing the cells with buffer only. In contrast, recovery to base line values was incomplete upon repeated exposure to CPP suggesting that the peptide is toxic in long-term applications. (Ziegler and Seelig 2007, *Biochemistry* 46, 8138-8145).

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Interaction of Verapamil with Lipid Membranes and P-glycoprotein. Connecting Thermodynamics and Membrane Structure with Functional Activity

Verapamil and amlodipine are calcium ion influx inhibitors of wide clinical use. They are partially charged at neutral pH and exhibit amphiphilic properties. The non-charged species can easily cross the lipid membrane. We have measured with solid state nmr the structural changes induced by verapamil upon incorporation into phospholipid bilayers and have compared them with earlier data on amlodipine and nimodipine. Verapamil and amlodipine produce a rotation of the phosphocholine headgroup away from the membrane surface and a disordering of the fatty acid chains. We have determined the thermodynamics of verapamil partitioning into neutral and negatively charged membranes with isothermal titration calorimetry. Verapamil undergoes a pK-shift of ΔpK = 1.2 units in neutral lipid membranes and the percentage of the non-charged species increases from 5% to 45%. Verapamil partitioning is increased for negatively charged membranes and the binding isotherms are strongly affected by the salt concentration. The electrostatic screening can be explained with the Gouy-Chapman theory. Using a functional phosphate assay we have measured the affinity of verapamil, amlodipine and nimodipine for P-glycoprotein, and have calculated the free energy of drug binding from the aqueous phase to the active center of Pgp in the lipid phase. By combining the latter results with the lipid partitioning data it was possible, for the first time, to determine the true affinity of the three drugs for the Pgp active center if the reaction takes place exclusively in the lipid matrix. (Meier *et al.* 2006, *Biophys. J*. 91, 2943-2955).

NMR-Analysis of Body Fluids (Metabolomics)

High resolution NMR analysis is used to elucidate the metabolic composition of blood plasma and urine. We are interested, in particular, in the influence of ultra-endurance exercise (over days and weeks) on the metabolism of the individual athletes (in collaboration with the Gesundheitszentrum St. Gallen and the University of Zürich). The NMR analysis is accompanied by anthropometric and bioelectric impedance measures to obtain additional information concerning changes in body composition such as fat mass, lean body mass and skeletal muscle mass.

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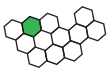

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Insights into functional protein evolution at atomic level

The primary goal of our research is to understand in detail the structure-function relationship of proteins as dynamic systems. We are mainly focused on intersubunit signaling linked to catalytic turnover, to explore the storage and delivery function of coiled-coil domains and to understand mechanisms of signal transduction via the extracellular matrix. To reveal the structural and functional basics of the underlying complexes, we use a combination of

– X-ray crystallography (in combination with SAXS and EXAFS) and solution NMR
– several biophysical techniques (e.g. CD-spectroscopy, D(S)LS, ITC and DSC)
– *In vitro* functional analysis (molecular biology and biochemistry)

Our long term goal is to develop a molecular understanding of target proteins at work. The research will provide new insights into basic mechanisms of how these proteins function, and applied science of great biotechnological and biomedical importance will be advanced.

Structural Insight into cofactor-dependent enzyme catalysis

Enzymatic function is tightly connected with the formation of transition states between enzyme and substrate to decrease the activation energy of the reaction. However, this is embedded in substrate attraction and product release. To establish coupling of these different functions, allosteric enzymes show a high degree of interactions amongst and within their components which can be described as molecular switcher connected via communication elements. A detailed structural understanding of these switch elements in enzyme catalysis will help to develop new approaches for understanding molecular details in enzyme chemistry and interdomain communication.

The tetrapyrrole ring in cofactors like haem, chlorophyll or coenzyme F430 is constructed from eight molecules of 5-aminolevulinate. In plants and some bacteria, this compound is synthesized by glutamate-1-semialdehyde aminomutase (GSAM), an a2-dimeric PMP/PLP-dependent enzyme of the a-family, with 433 residues per chain.The enzyme catalyzes the reaction:

1) E-PMP + glutamate-1-semialdehyde <-> E-PLP + 4,5-diaminovalerate

2) E-PLP + 4,5 diaminovalerate <-> E-PMP + 5-aminolevulinate

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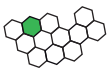

by an intramolecular exchange of amino and oxo functions and has no requirement for a-carboxylic amino or oxo acids to complete the reaction. Both subunits of the enzyme show asymmetry in cofactor binding and in the mobilities of the residues 153-181. In the unliganded enzyme, one subunit has the cofactor bound as an aldimine of PLP with the active site residue K273 and in this subunit the residues 153-181 are disordered. In the other subunit in which the cofactor is non-covalently bound as PMP, residues 153-181 are ordered. Furthermore, the conformation of residues 30-42 is slightly different in the two subunits. Since these two regions involve the active site and the substrate binding pocket this phenomenon must be related to the function. Analysis of the absorption spectra of the enzyme in solution during reduction show that half of the enzyme is reduced rapidly and the other half slowly, consistent with the existence of an asymmetric dimer and providing evidence for communication between the subunits.

Structure and Function of ECM proteins

Molecular studies of the extracellular matrix (ECM) are challenging because of the enormous complexity of its constituent protein and polysaccharide molecules and the multiplicity of interactions between them. These proteins generally are very large, almost invariably glycosylated, integral or membrane associated and often difficult to express in recombinant form. Fortunately, most ECM proteins are modular, i.e., composed of a limited set of relatively small modules. An intriguing feature of these domains is the fact that they are either involved in tandem formation within the same protein or in formation of highly visible protein complexes. However, relatively little is known about how these modules interact with each other or with ligand/ receptor molecules.

Alternative mRNA splicing is an important way to modulate the biological activity of a single gene. A well documented example is the extracellular matrix protein agrin where specific splice inserts in the C-terminal laminin-G-like domain (LG3) are required for induction of postsynaptic differentiation at the neuromuscular junction as well as the modulation of the binding to a-dystroglycan. To elucidate the structure-function relationship of LG3 a detailed analysis using NMR-solution and high-resolution X-ray studies was performed. Our findings show that the functional splice inserts adopt a highly flexible structure within the domain and become probably only structured upon binding to the yet unknown agrin receptor. The calcium-binding site seen in the crystal structures is highly conserved among agrin, neurexins and laminins, where it plays an important role for the binding to a-dystroglycan. Our results indicate that the calcium binding in LG3 plays an important role for the stabilization of the domain and prevents it from become partially unfolded.

Publications

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LSTF
Life Sciences Training Facility

The Life Sciences Training Facility is a platform for high-throughput DNA and RNA analysis

High Density Oligonucleotide Microarrays (GeneChips; http://www.affymetrix.com/) are well established tools for the genome-wide analysis of DNA mutations (SNP-Chips, Comparative Genome Hybridization), RNA concentrations (Expression profiling) and genome-wide protein-DNA interactions (ChIP-Chip assay). Novel generations of arrays enable scientists to study gene expression at the level of individual exons (all exon arrays) or independently of previous genome annotation (tiling arrays). The LSTF provides a state of the art infrastructure for all steps from sample processing and quality control to data analysis and interpretation.

The Microarray Information Management and Annotation System (MIMAS) was developed and published to provide life scientists with a flexible and GeneChip data storage and annotation platform. The system is accessible to members of the Swiss Array Consortium (SAC) via the internet web site at http://mimas.vital-it.ch/. The MIMAS software is available for free upon request. Users are invited to participate in training courses focussing on commercially available software solutions such as Agilent's GeneSpring and open source packages (CTWC, http://ctwc.bioz.unibas.ch/; ExpressionProfiler, http://ep.ebi.ac.uk/EP/). These courses are organised in cooperation with E. Domany (Weizmann Institute, Rehovot, Israel) and A. Brazma (EBI, Hinxton, UK).

N.B. The Life Science Training Facility was tranferred in 2007 to the faculty of psychology and is now headed by Prof. Andreas Papassotiropoulos. More information at http://www.biozentrum.unibas.ch/corelab/

Publications

Hermida, L., Schaad, O., Demougin, P., Descombes, P. & Primig, M. (2006). MIMAS: an innovative tool for network-based high density oligonucleotide microarray data management and annotation. *BMC Bioinformatics* 7, 190.

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From Proteomics to Phosphoproteomics: a daunting endeavour

Phosphorylation regulates a vast number of biological processes. Traditional biochemical and genetic analyses of phosphoproteins, and of the protein kinases and phosphatases that modify them, have yielded a wealth of information about signalling pathways. The availability of modern analytical technology has made it possible to study a large number of phosphorylation sites at once. In our lab, the focus in phosphoproteomics lies mainly on three levels. Firstly, phosphorylation usually occurs substoichiometrically and therefore efficient selection methods are required for collecting as many phosphopeptides as possible from cellular extracts. We considerably improved existing phosphopeptide selection by TiO2 to obtain highly enriched preparations from extracts of yeast and HeLa cells. Secondly, due to the preferential loss of phosphate during collision-induced fragmentation of phosphopeptides, a three step scanning procedure was set up to specifically detect phosphopeptides and concomitantly locate the site of phosphorylation. Thirdly, to track changes in the extent of phosphorylation that occur by a given treatment, the isotope-coded iTRAQ labelling reagent has been used in conjunction with software optimization to specifically allow the detection of reporter ions in a mass range hitherto impossible for ion trap instruments. Alternatively, SILAC is currently being investigated to track changes in phosphorylation in extracts of HeLa cells. In a collaborative effort with Mike Hall's lab, the methods described above are combined to obtain an as complete phosphoproteome map as possible of the Tor signalling complex in yeast and HeLa cells.

Substrate identification is crucial for obtaining a detailed molecular understanding of the full repertoire of the cellular functions a protein kinase exerts upon a given stimulus. Therefore, we apply proteomics tools to identify physiologically relevant substrates for the yeast Npr1 protein kinase (nitrogen permease reactivator). Mass spectrometric dissection revealed intriguingly complex phosphorylation events following nitrogen deprivation of yeast. Molecular analysis of a large number of phosphorylation sites indicates that phosphorylation modulates kinase stability rather than activity per se. With a modified KESTREL method we were able to show that Npr1 is a member of the family of basophilic protein kinases and a library of synthetic peptides revealed the strong preference for substrates with a basic residue at the P-3 position. Even though a *bona fide* substrate for Npr1 has yet to be found, the KESTREL method is continuously being refined in our laboratory. In parallel, by using bacterially expressed phosphorylation domains of Npr1 we are interested to track upstream protein kinases that are able to provoke such exceedingly complex regulation of Npr1 phosphorylation when changing the availability of the nitrogen source in yeast.

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TMCF
Transgenic Mouse Core Facility

The Transgenic Mouse Core Facility (TMCF) of the University of Basel provides transgenic and transgenic related techniques as a service to the researchers of the University of Basel.

Service
TMCF offers the injection of conventional DNA constructs and BAC's into the nuclei of fertilised oocytes, and carries out the injection of ES cells into blastocysts, and mouse line rederivation by embryo transfer. As a new additional service we are now offering the cryopreservation of mouse embryos including storage. TMCF provides material and methods for ES cell work. The aim is to work closely with the researchers and give technical support throughout the duration of an experiment. TMCF also evaluates the requirements and extends the services offered accordingly. The facility consists of an injection suite, a cryopreservation and mouse embryo thawing work station, lab space for general mouse embryo work, animal rooms, and a lab to carry out surgical procedures under sterile conditions, a molecular biology lab for quality control and sample preparation, and a mouse stem cell lab.

Staff
In 2007 TMCF was joined by Thomas Hennek, an experienced Microinjectionist, formerly with Artemis in Cologne and the Transgenic Facility of the University of Tübingen. Another new addition to the team is Désirée Gronostay, she joined to establish the Cryopreservation Service.

Advisory board
The TMCF Advisory board consists of the Professors Markus Rüegg, Yves Barde, and Rolf Zeller and has now been joined by Professor Bernhard Bettler. He is succeeding Rolf Zeller in chairing the Advisory Board for 2008.

Events
After the great success of the Minisymposium in 2005, TMCF organized a 2 day workshop discussing micro injection techniques and getting hands on experience using novel equipment and techniques.

Publications
One of the highlights was the publication of our first mouse ES cell targeting experiment, done with Jean Pieters: Jayachandran R, Sundaramurthy V, Combaluzier B, Mueller P, Korf H, Huygen K, Miyazaki T, Albrecht I, Massner J, and Pieters J, `Survival of Mycobacteria in Macrophages is Mediated by Coronin 1 Dependent Activation of Calcineurin`, *Cell*. July 13, 2007

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ZMB
Center of Microscopy of the University of Basel

The ZMB is a central service unit for microscopy that provides imaging support and training. The service covers Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and specialized light microscopy, such as Confocal Laser Scanning Microscopy (CLSM), Video- and Fluorescence Microscopy. Administratively, the ZMB is integrated into the Biozentrum. Nevertheless, its service covers the microscopy requirements of all the faculties of the University of Basel. Historically, it resulted from a fusion of the Interdepartmental Electron Microscopy unit of the Biozentrum and the REM-Laboratory of the University of Basel.

Services

The ZMB facilities comprise a Preparation Laboratory, two Transmission Electron Microscopes and three Scanning Electron Microscopes. It not only offers full microscopy service but also training for individuals to use the instruments independently in collaboration with the Biozentrum, that provides certain additional instruments, the ZMB service also includes training and technical support for Fluorescence - and Confocal Light Microscopy. Additionally, a Confocal Laser Scanning Microscope (CLSM) with image processing hard- and software is available on on request.

Courses

The ZMB organizes following training courses in microscopy:

- One week workshops in “applied microscopy for molecular biologists”
- One week workshops in “applied microscopy for organismic biologists”
- Two three-week workshops in microscopy for students in nanotechnology.

For further details on time schedule and registration, please contact the Units’ secretary.

Research

The ZMB collaborates with researchers and offers to take over the imaging part of their work. In addition, it continiously works on the development of new preparation methods and innovative instrumentation.

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BioPhIT (Biozentrum / Pharmazentrum Information Technology) and Bioinformatics System Administration

BioPhIT administers and maintains the central and local IT infrastructure provided to research and service groups throughout the Bio-/Pharmazentrum. In doing so, BioPhIT acts together with Bioinformatics System Administration and the recently founded Research IT to provide general assistance when dealing with IT related aspects of research projects.

User Support

BioPhIT currently supports about 400 Active Directory (AD) Windows users working on 350 highly integrated Windows AD desktop systems. In addition, about 100 standalone desktop and notebook machines (Windows and Macintosh) plus 30 specialized workstations mostly attached to data acquisition instruments are supported and maintained.

Biozentrum users can contact BioPhIT through the web using a ticketing system or by phone via a central support hot line. The ticketing system was upgraded to a new version now offering enhanced automated support and reporting features to the user. An obligatory user introduction for new members of the Bio-/Pharmazentrum was initiated to provide basic knowledge about IT infrastructure usage at the institute right from the start.

Windows ADS Environment

BioPhIT administers central file server storage offering HOME and Group drives to all AD users, either connecting from Windows or Macintosh computers. HOME, Group drives and the central user profiles carrying personal settings of AD users were migrated onto the central storage system of the University Computing Centre (URZ) and thereby expanded to 20 TB total capacity.

A new system for central software deployment was implemented. This system allows remote software installation or upgrading of existing programs tailored to the requirements of individual research groups.
Calendaring System

In 2006, BioPhIT introduced Oracle Collaboration Suite (OCS) and offered this groupware to group leaders and secretary‘s offices at the Biozentrum as shared, platform independent calendaring system. Meanwhile, it is successfully utilized by more then 70 users.

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New since 2007
Research IT
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BioPhIT
Biozentrum/Pharmazentrum Information Technology and Bioinformatics System Administration

IT Board

To further improve coordination of IT activities within the Biozentrum and to better communicate them to the group leader conference an IT board was launched. All Core Programs (CP) and Focal Areas (FA) of the Biozentrum are represented by one member within the committee. Current members are:
S. Arber (FA Neurobiology), C. Dehio (FA Infection Biology), A. Spang (FA Growth & Development), S. Grzesiek (CP Structural Biology & Biophysics), T. Schwede (CP Computation & Systems Biology), R. Jenni (IT-Coordinator)

Bioinformatics System Administration

Bioinformatics system administration manages the working environment of the CP "Computational & Systems Biology". This includes a 246 core Linux based high-performance compute cluster attached to several login, file and database servers, about 18 TB storage, centralized management of software plus automated update procedures for biological DNA and protein databases. The setup is completed by a PC-Grid infrastructure connecting PC's of the Biozentrum, University classrooms and the Friedrich-Miescher Institute to a large virtual computer. Bioinformatics system administration also maintains the necessary infrastructure for various web pages including scientific web services.

The cluster infrastructure (compute nodes and file storage) was rotationally replaced and further expanded to have the continuing ability of providing sufficient computational resources. Unfortunately, expansion was limited and delayed by the availability of infrastructural resources (mainly cooling capacity). Particularly with regard to current hardware evolution and foreseeable needs of upcoming high-throughput data generating techniques, providing sufficient cooling capacity for high-performance computing will become a major concern.

A high-performance file system (General Parallel File System, IBM) was implemented.

The previously mentioned PC grid (United Devices) was successfully used within the "Dengue Docking" project to identify new potential lead compounds against dengue fever by protein-ligand docking.
An independent surveillance system for the Bioinformatics and BioPhIT IT infrastructure was implemented. The system steadily monitors all vital functions including environmental conditions. In case of breakdown issues the system is capable of sending automated notification alerts via SMS.

Analysis Platform for Mass Spectrometry Data

A data analysis platform for the mass spectrometry service facility at the Biozentrum was build up. In a first step an 8-nodes SEQUEST cluster was implemented. Later on, an 8-CPU Phenyx environment was added to expand the setup.

Research IT

2007 saw the establishment of Research IT at the Biozentrum. As modern molecular biology increasingly relies on computational tools and infrastructure, Research IT is an in-house function to build bridges between research scientists, computational biologists and infrastructure providers, such as the university computer center (URZ).

Research IT supports research projects on a per-project basis: In the planning phase, we help to align the project's requirements with the existing environment and infrastructure. Later on, we stay involved throughout the project lifetime. Research IT activities span a wide range from infrastructure coordination to software evaluation and custom software development.

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