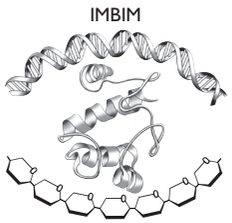
ANNUAL
REPORT
2012



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Medical Biochemistry
and Microbiology
IMBIM

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Department of
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DEPARTMENT OF

MEDICAL BIOCHEMISTRY

AND MICROBIOLOGY

ANNUAL REPORT

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An Icelandic horse in flying pace homozygous for the Pacemaker mutation (Photo: Freyja Imsland, IMBIM).

A pacing horse moves the two legs on the same side of the body in unison. Leif Andersson and his group have discovered a nonsense mutation in the novel DMRT3 transcription factor that has a strong impact on the control of gait in horses. All tested horses that can perform the flying pace is homozygous for this mutation. The mutation is also associated with the ability to perform different types of ambling gaits (running walk) and with performance in harness racing. Publication: Andersson LS, Larhammar M, Memic F, Wootz H, Schwochow D, Rubin CJ, Patra K, Arnason T, Wellbring L, Hjälm G, Imsland F, Petersen JL, McCue ME, Mickelson JR, Cothran G, Ahituv N, Roepstorff L, Mikko S, Vallstedt A, Lindgren G, Andersson,L, Kullander K. 2012. Mutations in DMRT3 affect locomotion in horses and spinal circuit function in mice. Nature. 488:642-646.

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INTRODUCTION

I would like to begin by thanking Kristofer Rubin who has functioned as the acting chairman for IMBIM during the last four years. During his time as chairman, IMBIM has undergone a major transformation in size and financial turnover. Thus, the number of people employed at IMBIM has increased to around 165. Counting also post docs and students the figure of people working and studying at IMBIM increases to around 230. The financial turnover for IMBIM has also undergone large transformations during recent years and doubled since 2005. Much of this has happened under Kristofers time at the rudder. We are most grateful for the work and dedication that he has devoted to IMBIM over the years and it is our hope that he will continue to be an active part of IMBIMs future development although he now will spend most of his time in Lund. From a personal point I sincerely hope that we will be able to fill the void that is left after that he stepped down as the chairman at IMBIM. Good luck Kristofer and don't be a stranger.

It is with a great pleasure I can follow the construction of the new SciLife building (Navet), at a front row seat right from my office window. It is with a remarkable speed that the construction progresses. The building is expected to be finished before the end of 2013. Since IMBIM functions as a host for the SciLife project with the director and the administrative functions coupled to IMBIM we see this project as an important opportunity, not only for Uppsala and the Swedish research community but also a chance of a lifetime for all personnel working at IMBIM. Seize the chance.

Prizes and Awards:

Several scientists at IMBIM have received prestigious awards during 2012. The most notable was the election of Professor Leif Andersson as a foreign associate of the National Academy of Sciences (USA). To become a member of this prestigious academy is something that is not bestowed on everyone. The Academy has only 430 foreign members whereof only eight are from Sweden. Leif is the only Uppsala researcher that currently is a member. Professor Dan Andersson was elected fellow by the American Society of Microbiology in 2012. This is also a society with a select number of members. Thus, there are only six Swedish scientists that currently are members of this prestigious Academy. Professor Kerstin Lindblad-Toh was also elected a member to the Swedish Royal Academy of Sciences. In addition, Leif Andersson and Kerstin Lindblad-Toh became Wallenberg Scholars during 2012. Leif Andersson also received the Hilda and Alfred Eriksson's prize in medicine by the Royal Swedish Academy of Sciences. Leif Andersson, together with Lisa Andersson and Gabriella Lindgren, were also named the Innovator of the year in Uppland by ALMI. We also congratulate Assistant Professor Jenny Hallgren-Martinsson who is the first to receive the newly established Lennart Philipson award, which is given to young scientists to help them establish an independent research carrier. Finally I would like to mention that Professor Göran Magnusson during 2012 received the Gustaf Adolfs medal in Gold from Uppsala University for his long lasting work for the University at many important positions. Göran Magnusson retired in January 2013 but continues his important work now as a Senior

Professor functioning as a unifying link for the organization of new research facilities at our Faculty.

Teaching:
Teaching of undergraduate and graduate students is a primary undertaking for IMBIM. During 2012 a total of sixteen students were registered as PhD students and twelve students received their doctoral degree and one student a licentiate degree. The teachers at IMBIM does an excellent job something that is illustrated by the fact that both Professor Birgitta Tomkinsson and Professor Erik Fries have received pedagogic prizes from the Medical and Biomedical students during 2012. IMBIM congratulates both prizewinners for their well-deserved recognition.

Scientific Highlights:
The work at IMBIM has also been the focus in multiple press releases and newspaper articles describing the people and the research they have been done at the Department. This type of media attention is important to increase the public understanding of basic science and to improve the attitude towards science in general. During 2012 more than 100 scientific articles were published with scientists from IMBIM involved. More than 10% of these were published in top journals like Nature, Science and PNAS.

Ongoing research projects at IMBIM are summarized later in this annual report. Below are a summary of three of the studies that received large media attention during 2012.

Leif Andersson and his group of collaborators published in Nature a study were they identified a gene (DMRT3) controlling the pattern of locomotion in horses. All horses can walk, trot and gallop but horses carrying the specific mutation can also perform ambling gaits, also called running walk, pace and it was shown that the mutation was also favorable in horses used for harness racing. The causative mutation is a nonsense mutation in DMRT3 that leads to the expression of a truncated form of the protein. Studies in the mouse revealed that DMRT3 is expressed in a specific subset of interneurons in the spinal cord that make direct connections to motor neurons colons controlling muscle contractions. Furthermore, characterization of a DMRT3 knockout mice confirmed that this gene has critical role in the neuronal circuits coordinating movements. The study is an advance in our understanding how locomotion is coordinated by neural circuits in the spinal cord probably in all vertebrates including the human (***Nature* 488, 642-6546**).

Joakim Näsvall and Dan Andersson published a paper in Science describing a novel mechanism for how new genes can evolve in bacteria and eukaryotes. These results open up a new field for experimental study of evolution of novel gene functions in real time (***Science* 338:384-387**).

Kerstin Lindblad-Toh and coworkers published in Nature the stickleback genome. The paper describes the identification of hundreds of selective sweep signals related to adaptation to a freshwater environment. It also demonstrates that the majority of mutations responsible for this adaptation process are located in non-coding sequence suggesting that gene regulation

plays a more important role than actual protein changes. It also highlights an important role for genome rearrangements in evolution (***Nature* 484, 55-61**).

The administrative and technical staff at IMBIM does a fantastic job to support the researchers working at IMBIM. The service they provide is of highest standard making life bearable as a chairman for this large unit. Without the effective support functions at IMBIM, the output in terms of research and teaching would deteriorate rapidly. Unfortunately, several of the staff members will retire during 2013, causing both a challenge and opportunity for the Department to further reform its ambitions and goals.

IMBIM also congratulates Jin-Ping Li who was promoted to Professor and Per Jemth who was appointed as "universitetslektor" at Uppsala University during 2012.
Finally, I would like to thank all of those who left IMBIM during the past year for your involvement in building up this fantastic scientific environment. I wish you all the best for the future and hope that you only have fun memories from your time at IMBIM. At the same time I welcome all new students/scientists etc. to this exciting environment and hope that you will actively contribute to the further development of this Department.

Uppsala February, 2013

Göran Akusjärvi
Chairman

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Birgitta Tomkinson
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Jessica Bergman
Dan Andersson
Ruslan Georgiev

SCIENTIFIC PRESENTATIONS

COMPARATIVE GENOMICS

Leif Andersson, Kerstin Lindblad-Toh, Pernilla Bjerling, Manfred Grabherr, Patric Jern, Gerli Rosengren-Pielberg, Matthew Webster

Comparative genomics is of crucial importance to unravel gene function and regulation. We are using domestic animals and other model organisms (*Schizosaccharamyces pombe* and mouse) to study genotype-phenotype relationships, gene regulation, chromatin organization and epigenetics.

Functional genomics in domestic animals. Domestic animals constitute a unique resource of genetic diversity due to their long history of selective breeding. We use a variety of domestic animals, including chicken, dogs, horse, pig, and honeybee, for in-depth studies of genes underlying both monogenic and multifactorial traits, as well as diseases of human relevance. We use both pedigree-based analysis and genome-wide association studies combined with high-throughput genomics and functional studies to achieve these goals.

Genome evolution. We use comparative analysis to identify functional elements in the human genome and those of model organisms to study the evolution of these elements and other genomic sequences. For example, comparison across 29 mammals identifies 3.6 million elements of which we can suggest a function for ~60%. Evolutionary analysis also identifies lineage-specific selection and innovation of both protein coding and regulatory elements. Furthermore, analysis of genetic variation within species enables us to identify regions targeted by selection, and to understand the mechanisms and evolution of recombination.

Chromatin organization and epigenetics. We investigate how transcription factors and silencer proteins influence the epigenome, both by changes in chromatin modifications and organisation of the chromatin within the cell nucleus. More specifically, we are investigating the newly identified transcription factor ZBED6, its mechanism of action and its possible role in human diseases. In addition, we are using the *S. pombe* model system to understand the complex relationship between the spatial organisation of the cell nucleus and the regulation of genome function.

Host – retrovirus evolution. Retroviruses have successfully colonized vertebrate genomes for millions of years as endogenous proviruses (ERVs). This genomic record provides a unique perspective on host-virus relationships. We deploy a bioinformatics approach to identify ERVs in genomic sequences of domestic animals and other vertebrates to further our understanding of retrovirus evolution and effects of retroviruses on host genome function.

Computational biology. Computational biology plays a key role for all of the above research areas. To support novel research questions and new data types we develop new algorithms and analysis methodologies, and we make the software publicly available for researchers around the world. These include tools to align entire genomes to each other to determine their relationships on a highly localized level, to *de-novo* assemble transcripts from RNA-Seq data, and to identify signals of selection within populations on a genome-wide scale.

FUNCTIONAL GENOMICS IN DOMESTIC ANIMALS

Leif Andersson

The overall objective of the group is to use genetics and genomics to advance our understanding of the genetic mechanisms underlying phenotypic variation. We are using domestic animals models because domestication and animal breeding have caused major changes in many phenotypic traits. This gives a unique opportunity to unravel the genes underlying phenotypic variation. The research includes genetic studies of both monogenic traits, like inherited disorders and coat colour, and multifactorial traits, like muscle development, fat deposition, autoimmune disorders and general disease resistance. The research projects involve genetic studies in chicken, horses, pigs, rabbit, Darwin finches and Atlantic herring. The ultimate goal of the research is to identify the genes and mutations affecting a certain trait and thereafter study the mechanism as well as the biological and medical significance of the genes and regulatory elements affected by the observed mutations. An important tool in the current research is to use whole genome resequencing to detect signatures of selection.

Members of the group during 2012
Leif Andersson, professor, group leader
Ben Dorshorst, post-doc (until September 2012)
Jonas Eriksson, PhD student (until June 2012)
Anna Golovko, researcher
Rajesh Gupta, researcher
Freyja Imsland, PhD student
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Khurram Maqbool, PhD student, (associated with Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences)
Doreen Schwochow, PhD student, (associated with Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences)

International exchange during 2012
Chao Wang, Huazhong Agricultural University, (visiting student during nine months)
Miguel Carneiro, University of Porto, (visiting researcher during two months)

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GENETIC ANALYSIS OF DIVERGENT INTERCROSSES OF CHICKEN

Ben Dorshorst, Jonas Eriksson, Freyja Imsland, Carl-Johan Rubin, Chao Wang, Leif Andersson

We have in collaboration with Prof. Per Jensen (Linköping) and Prof. Paul Siegel (Blacksburg, USA) developed two unique resource pedigrees for genetic dissection of multifactorial traits. One of these pedigrees was generated by crossing a single Red junglefowl male with females from a line of White Leghorn selected for egg production. The populations differ markedly in growth, fertility (number of eggs and size of eggs), behaviour and body composition (fat vs. protein content). The other intercross involves two lines (High and Low) that have been divergently selected for growth for more than 40 generations. The selection response has been remarkable and the 8-week weight shows approximately a 9-fold difference between lines, about 1.8 kg for the H line in contrast to about 0.2 kg for the L line. The two lines show a dramatic difference in appetite (hyperphagia vs. anorexia) and the body composition has been altered with the H line developing obesity. The HxL intercross is a unique resource for understanding the genetic regulation of growth, appetite and obesity. Both pedigrees comprise almost 1,000 animals from three generations. This experimental design gives an excellent power in the genetic analysis. A broad collection of phenotypic data and DNA samples has been obtained from all animals. Our strategy is to combine genetic marker and gene expression analysis for gene discovery. The analysis of the Red Junglefowl intercross has localized more than 40 quantititave trait loci (QTL) controlling growth, body composition, fertility, egg weight, behaviour and bone density. Using these resources we have identified the genes causing dominant white colour, dominant black colour, silver plumage colour, yellow skin and Pea-comb. More recently we have employed next-generation sequencing to resequence the chicken genome from different populations with the aim to reveal loci that have been under strong selection during chicken domestication.

The project is carried out with collaborators at Uppsala university, Linköping university, Swedish University of Agricultural Sciences, INRA (France) and Virginia Polytechnic Institute (Blacksburg, USA).

ZBED6 – A NOVEL MAMMALIAN TRANSCRIPTION FACTOR ORIGINATING FROM A DNA TRANSPOSON

Lin Jiang, Shady Younis, Ola Wallerman, Rajesh Gupta, Elisabeth Sundström, Carl-Johan Rubin, Leif Andersson

A single nucleotide substitution in intron 3 of *IGF2* in pigs abrogates a binding site for a repressor and leads to a three-fold upregulation of *IGF2* in skeletal muscle. The mutation has major effects on muscle growth, size of the heart and fat deposition. We have identified this repressor and found out that the protein, named ZBED6, is a previously unknown protein, specific for placental mammals and derived from a domesticated DNA transposon. ChIP-sequencing using C2C12 cells identified about 2,500 ZBED6 binding sites in the genome and the deduced consensus motif gave a perfect match with the established binding site in *Igf2*. Genes associated with ZBED6 binding sites showed a highly significant enrichment for certain GeneOntology classifications including development and transcriptional regulation. The phenotypic effects in mutant pigs, the extreme sequence conservation, its nucleolar localization, the broad tissue distribution and

the many target genes with essential biological functions suggest that ZBED6 is an important transcription factor in placental mammals affecting development, cell proliferation and growth. More recently we have revealed that ZBED6 has a significant role for transcriptional regulation and differentiation in pancreatic beta cells. A broad research program involving functional assays and mutation screenings in humans has been initiated to study the biological and medical significance of ZBED6. This includes the generation of *Zbed6* knock-out mice and the first *Zbed6* +/- mice were born in December 2012.

The project is carried out in collaboration with researchers at Uppsala University, Swedish University of Agricultural Sciences and Karolinska Institutet.

DETECTING SIGNATURES OF SELECTION USING WHOLE GENOME RESEQUENCING

Alvaro Martinez Barrio, Miguel Carneiro, Sangeet Lamichhaney, Khurram Maqbool, Marta Promerova, Nima Rafati, Carl-Johan Rubin, Görel Sundström, Leif Andersson

Next generation sequencing offers the possibility to carry out whole genome resequencing of large genomes, like the human. We have pioneered the use of using pooled samples to detect signatures of selection when comparing different populations that has been exposed to different selection pressures. Our first application of this approach involved whole genome resequencing of eight different populations of domestic chicken (four broiler populations and four layer populations), a pool of red junglefowl birds and the single red junglefowl female that was previously used to produce a draft genome assembly for the chicken. The project was very successful and resulted in (i) the detection of more than 7 million single nucleotide polymorphism, (ii) 38 loci with strong signatures of selection and (iii) almost 1300 deletions with a high frequency in at least one population. We have also applied the method in pigs and Atlantic herring. We are currently applying this approach to the Atlantic and Baltic herring, wild and domestic rabbit, and Darwin finches.

GENETIC ANALYSIS OF THREE CHICKEN MODELS FOR AUTOIMMUNE DISORDERS IN HUMANS

Susanne Kerje, Leif Andersson

We have intiated cross-breeding experiments and genome scans for three lines of chickens representing novel models for three autoimmune disorders in humans, Hashimoto's thyroiditis, systemic sclerosis and vitiligo. The Obese strain (OS) chickens develop a spontaneous autoimmune thyroiditis closely resembling Hashimoto's thyroiditis in human. The strain was established in the 1960's and has been widely used as an animal model to reveal various aspects of the disease. The University of California at Davis line 200 (UCD200) chickens develop an inherited syndrome with features very similar to human systemic sclerosis including fibrotic destruction of the skin and internal organs. Finally, the Smyth line (SL) represents an animal model for vitiligo in which 70-90% of the birds express a post-hatch autoimmune destruction of melanocytes leading to feather de-pigmentation at 6–14 weeks of age. Interestingly the incidence of vitiligo is dramatically increased (from ~15% to ~85%) after immunization with a Herpes virus vaccine. Virus infections are generally believed to trigger autoimmune disorders in humans. The

intercross pedigrees are used for genome scans with the ultimate goal of identifying genes underlying these autoimmune disorders. The identification of disease-related genes will lead to a better understanding of pathogenesis, as well as of general mechanisms underlying autoimmune diseases, thus facilitating the development of better diagnostic, prognostic and therapeutic tools. The work is carried out in collaboration with Drs. Olov Ekwall and Olle Kämpe at Department of Medical Sciences and Dr. Örjan Carlborg at SLU.

MOLECULAR CHARACTERIZATION OF THE MUTATION AND MECHANISM CAUSING GREYING WITH AGE IN HORSES

Anna Golovko, Elisabeth Sundström, Lin Jiang, Leif Andersson

Grey is a dominant coat colour mutation that is common in horses and found in a variety of breeds including Arabian horses, Lippizzaner horses, Thoroughbreds, Swedish Warmblood and Icelandic horses. A grey horse is born coloured (e.g. bay, black or chestnut) but for each year it becomes gradually greyer and eventually all hair become completely white. A remarkable feature of this coat colour variant is that there is a very high incidence of melanomas in old grey horses. It has been estimated that ~80% of grey horses older than 15 years have melanomas whereas this is a very rare condition in horses with other colours. Thus, the identification of the *Grey* mutation provides an opportunity to generate new basic knowledge about tumour development of melanocytes. The causal mutation is a 4.5 kb duplication located in intron 6 of *Syntaxin17*. We also demonstrated that this is cis-acting regulatory mutation that upregulates both *Syntaxin17* and the neighbouring gene *NR4A3* encoding an orphan nuclar receptor. We are currently exploring the mechanism leading to premature greying and melanoma development. The work is carried out in collaboration with researchers at University of Natural Resources and Applied Life Sciences, Vienna.

CHROMATIN DYNAMICS

Pernilla Bjerling

The basic unit of chromatin is the nucleosome, consisting of a core of histone proteins that the DNA is wrapped around. The chromatin is constantly undergoing dynamic changes adjusting the transcriptome during development and as a response to environmental stimuli. We study both transient and stable, or epigenetic, changes. Epigenetic changes in the genome is believed contribute significantly to several diseases like cancer, diabetes type II and obesity. We study two aspects of chromatin dynamics, changes in chromatin and the influence of subnuclear localisation on the expression status of a gene. By using fission yeast, *Schizosaccharomyces pombe,* as a model system we can learn more about what determines the epigenome and how switches between different types of chromatin can occur.

Members of the group during 2012
Pernilla Bjerling, group leader
Alejandro Rodriguez, post-doc
Daniel Steinhauf, PhD student

Projects students during 2012
Yeasmeen Ali
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Publications 2010 to 2012

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FORMATION OF REPRESSIVE CHROMATIN

Daniel Steinhauf and Alejandro Rodriquez

In *Schizosaccharomyces pombe* there are three main regions where a special form of transcriptionally repressed chromatin, named heterochromatin, is formed; the pericentromeric region, the subtelomeric region and in the mating-type region. The

heterochromatin constitutes relatively stable structures in the cell nucleus and might therefore contribute to the organisation of the interphase genome and also to the fine-tuning of gene expression. The molecular characteristic of heterochromatin is conserved between fission yeast and human with low acetylation levels of the histones and methylation of lysine 9 at histone H3 (H3K9Me2/3). The methylation is made by histone methyltransferase, SUV39H among others in humans, and Clr4 in *S. pombe*. The H3K9Me2/3 modification creates a binding site for chromodomain proteins, HP1 in human, and Swi6, Chp1 and Chp2 in *S. pombe*. Moreover, Clr2 is crucial for heterochromatin formation in *S. pombe*, yet very little is known about the function of Clr2 (Bjerling et al, 2004). To find out more about the mechanism of action of Clr2, a bioinformatics approach that revealed three conserved motifs in the Clr2 protein as been undertaken. We have introduced point mutations changing the codons of conserved amino acids at the endogenous *clr2*$^{+}$ locus, and several of these mutations display silencing defects. Now we want to understand the effect of the mutations, perhaps they cause a disruption of the SHREC complex or disables Clr2: s association to chromatin.

MOLECULAR FUNCTION OF ZBED6

Daniel Steinhauf

ZBED6 is a recently discovered transcription factor unique to placental mammals. It was discovered because it acts as a repressor at the IGF2 locus. A point mutation disrupting its target site in IGF2 in domestic pigs leads to a three-fold upregulation of IGF2 expression and increased muscle growth. Interestingly, ZBED6 has evolved from a domesticated transposon and belongs to a family of related transcription factors. However, the molecular mechanism of this family of transcription factors is more or less uncharacterised. In this project the aim is to find interacting partners to ZBED6 using a yeast 2-hybrid approach and to perform a deletion study to find functionally relevant parts of the protein. The project is carried out in collaboration with Leif Andersson and his group.

CHROMATIN DYNAMICS DURING NITROGEN DEPLETION

Alejandro Rodriguez

There are several clusters of genes in *S. pombe* that are upregulated early during nitrogen starvation. We have labelled two of these loci using the *lacO*/LacR-GFP strategy, allowing for live cell analysis. During normal growth conditions the gene clusters localise to the nuclear periphery at the opposite side of the nucleus as compared to the spindle pole body (SPB). Already 20 minutes after nitrogen depletion drastic changes in subnuclear localisation of the two loci are observed, away from the nuclear membrane towards the nuclear interior (Alfredsson-Timmins et al. 2009). We are currently investigating whether the movement is crucial for induction of the genes by targeting the gene clusters to the NM preventing the movement. Moreover, by expression profiling we have identified 118 genes that are upregulated after 20 minutes of nitrogen starvation. In addition, we have done genome-wide Chromatin Immunoprecipitation (ChIP) CHIP assays to detect changes in nucleosome occupancy genome wide during nitrogen starvation. We find that nucleosomes are lost from all of the induced genes both at the promoter and in the coding region (Kristell et al. 2010). In addition the gene clusters that change sub-nuclear position during

nitrogen starvation display a drastic loss of nucleosomes over the gene bodies. Now, we would like to understand the regulation and the biological significance of this nucleosome loss.

HiSTIDINE KINASES AS DRUG TARGETS IN CANDIDA

Pernilla Bjerling

Several species of the pathogenic yeast *Candida* normally grow on the skin of humans and only people with a lowered immuneresponse, like immunosuppressed patient undergoing transplantation or AIDS patients, suffer from *Candida* infections. The drugs against *Candida* frequently give strong side effect so improved formulas would be of great importance. There are primarily two species that causes infections, *Candida albicans* and *Candida glabrata*. Bacteria, plant and yeast have on their cell surface histidine kinases that act as environmental sensors not found in higher eukaryotes and therefore they are promising drug targets. In *Candida albicans* the histidine kinases are important for the transition between yeast (unicellular) to hyphal (multicellular) growth and hence an important virulence factor, since this transition need to occur in order for Candida to penetrate the human skin. Therefore it is of interest to get drugs that prevent the formation of the virulent form of the yeast, rather then drugs that would just inhibit growth. This project aims to set up a drug screen against the histidine kinases in the two *Candida* species.

EVOLUTIONARY BIOINFORMATICS AND COMPUTATIONAL BIOLOGY

Manfred G. Grabherr

In the wake of novel sequencing technologies, the past decade has brought with it a wealth of genomic data, providing an in-depth view on the genetic basis for life. However, many important questions remain unanswered, both with respect to the evolutionary forces shaping the coding regions of genomes as well as the functional relevance of non-coding and intergenic regions. The Grabherr Group is thus centered on developing and applying computational algorithms and methods to explore sequence data and answer a broad range of biological questions.

One of the corner stones of our work is the use of *transcriptomics*, i.e. the analysis of the repertoire of transcripts that are expressed in a cell or sample and/or under certain conditions. Such data can provide valuable insights into the function of genes as well as help guiding the search for unknown genes or understudied genomic regions. In addition, we are using *comparative genomics* to study the variation within populations as well as across species to understand how speciation may occur on a genetic level or to trace the evolutionary trajectory of genes, such as those implicated in diseases. Perhaps most importantly, we work in close collaboration with a number of other research groups to explore the interplay between regulatory mechanisms and expression, functional characterization of transcripts, and meta-transcriptomics studies.

To help us in our research, we are developing algorithms and methods for whole-genome comparisons, protein alignments, detecting local changes in genomic phylogeny, and gene annotation and characterization.

Members of the group during 2012
Manfred G. Grabherr (group leader)
Neda Zamani (post-doc)
Marc P. Höppner (post-doc)
Henrik Lantz (BILS)

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EVOLUTION AND COMPARATIVE GENOMICS OF NON-CODING RNAS

Marc P. Höppner

Non-coding RNAs (ncRNA) are an abundant and diverse class of transcripts, found across the entire tree of life. Apart from their hallmark characteristic – the lack of protein-products - the different classes of ncRNAs are involved in a wide range of activities, including the regulation of gene expression or protein synthesis. However, despite their central role in molecular biology, their evolution or role in genome function and organization still remain poorly understood.

During the past year, we conducted several studies on different aspects of non-coding RNA evolution. Specifically, we published an in-depth reconstruction of the RNA inventory across all domains of life, showing that different domains are characterized by a discrete and unique repertoire of RNAs, whereas only a minor set is clearly shared across all cellular life, compatible with the emergence in a primordial 'RNA world' (Hoeppner, Gardner et al. 2012). In addition, we analyzed the intra- and inter-genomic dynamics of small nucleolar RNAs (snoRNAs) across the major kingdoms of eukaryotes, showing that a substantial number of extant snoRNAs are traceable to the last eukaryotic common ancestor (LECA), but that their genomic organization is transient (Hoeppner, Gardner et al. 2012; Hoeppner and Poole 2012). This finding provides both a comprehensive view on the history of this ncRNA family, but also informs discussions on the origin of introns as by the 'Introns-First theory'.

Over the next years, we plan to further pursue these questions using next-generation sequencing data from across the tree of life, especially the understudied but important eukaryotic kingdom of amoebozoa (with Prof. Fredrik Söderbom, ICM).

A COMPREHENSIVE CATALOGUE AND CHARATERIZATION OF TRANSCRIBED FEATURES FOR THE GENOME OF THE DOMESTIC DOG, *Canis familiaris*.

Marc P. Höppner

The domestic dog, *Canis familiaris*, is susceptible to a number of human diseases, including cancer and cardiomyopathy, and is as such a well-established model system. In collaboration with Kerstin Lindblad-Toh and her groups at IMBIM and the Broad Institute, we describe an improved genome build, canFam3.1, which now covers 99,8% of the euchromatic portion of the genome - on par with human or mouse. In addition, we generated a comprehensive and complex feature map for this improved build based on strand-specific RNA-sequencing data generated from 10 tissues and two different library preparations, polyA+ and DSN. This data set complements and greatly expands existing annotations, providing a rich inventory of transcribed elements that serve as functional candidates for future disease association studies. Among the tens of thousands of previously unknown regions, we find 5,200 new candidate proteins, 4,200 novel antisense elements, 4,900 putative lincRNAs, but also over 61,0000 intergenic transcripts of unknown function. Among the latter, many loci are expressed in a sample- rather than tissue-specific manner, compatible with a highly regulated activity. Our findings indicate that, if these novel loci are essential to the organism, their function is likely universal in all tissues. This property makes these transcripts especially attractive as candidates for experimental follow-ups in disease association studies, for example cancer, which can arise in very different tissues.

TIME-EFFICIENT PROTEIN ALIGNMENT

Neda Zamani

Next generation sequencing technologies are routinely generating increasing amounts of data, with this trend set to exacerbate by introduction of even more cost effective sequencers. This has lead to two main implications: (**a**) Relatively small laboratories can now conduct large-scale studies, with analyses that rely heavily or even exclusively on RNA sequencing becoming increasingly popular; but also, (**b**) available databases that store newly discovered nucleotide and protein sequences are growing with each new study. Many studies require characterizing novel sequences by homology search against known databases and as it is desirable to perform these comparisons against databases comprising all known sequences in order to avoid the propagation of erroneous annotations across species, this task is becoming the bottleneck in of such analyses.

To address the aforementioned problem, we have designed a new protein alignment program that supports high-throughput database searches by running 10-100 times faster than *BlastP*. Initial experiments show that the speedup is achieved while maintaining comparable specificity and with only minimal loss in sensitivity. The cost, however, lies in increased memory usage, which we consider a reasonable trade-off, since high-memory compute servers have become very affordable.

WHOLE GENOME COMPARATIVE FRAMEWORK

Neda Zamani

Comparative genomics provides a powerful means for establishing relationships between gene function and location in a variety of different organisms and gaining insight into conservation of functional elements and evolutionary histories. Related species often preserve the order and orientation of genes and other features over long genomic regions, which, in absence of large-scale duplications and deletions, allows for identifying orthologous sequences. While pairwise synteny aligners exist, the fast growing pool of available genomes, brought about by the increasing rate at which new genomes are being sequenced, prohibits the generation of all-to-all pairwise mapping.

We have thus designed and implemented a Rapid Universal Mapper, Rum, which infers sequence correspondence by walking along a graph of alignments. For example, if a region in genome A syntenically corresponds to genome B, and the region in genome B corresponds to genome C, which in turn is syntenic to genome D, then this allows for estimating a genomic interval in genome D that is orthologous to the sequence in genome A. We thus perform a re-alignment in two steps: (i) a fast heuristic search using cross-correlation, which finds the approximate coordinates, and (ii) an optimal local alignment to determine the exact boundaries of genomic features. Subsequently, entire gene annotations from many species can be accurately mapped over without the need to re-compute genome-wide alignments, even if the pairwise alignments between these genomes do not exist. In only a few hours of runtime, Rum allows for mapping millions of genomic features onto a mammalian-sized genome.

ANNOTATION OF EUKARYOTE GENOMES

Henrik Lantz

With the increased amount of *de novo* assembled genomes being produced, there is also an increased need for careful and scientifically sound annotation. In particular for genomes where there is no closely related reference genome available, the annotation procedure is time consuming and requires expert knowledge. These projects aims to supply that knowledge in collaborative efforts together with genome projects in Sweden and elsewhere. Each annotation project is, depending on the organism and type of data available, unique and we adapt the methods used for each project as needed.

Two projects are currently underway. The first is a large-scale project on the annotation of the crow genome, run together with the group of Jochen Wolf at the Evolutionary Biology Center, Uppsala University. Here, there is a large amount of rna-seq data available and our pipeline heavily relies on de novo assembled transcripts. The genome is also largely syntenic with the zebra finch genome, and this allows us to lift over the annotation from the zebra finch to the crow for comparison purposes and for functional annotation. The lift-over of an annotation from a closely related organism like this is made possible by software developed in the group.

The other project is a collaboration with the Department of Microbiology, SLU, Uppsala concerning the annotation of a highly xerotolerant fungus. Due to a lack of high quality RNA-seq data, this annotation pipeline instead relies on protein alignments and *in silico* constructed gene models, with functional annotation added using a blast-based approach.

THE COMPOSITION AND POPULATION DYNAMICS OF FUNGAL ENDOPHYTES IN THE NORWAY SPRUCE *Picea abies*.

Henrik Lantz, Marc Höppner, Manfred Grabherr

Endophytic fungi are beneficial to their plant hosts and can increase growth, stress tolerance, and provide protection by producing toxins. The Norway spruce, *Picea abies*, a conifer that keeps its needles for several years is a preferred target for colonization. However, very little was known with regards to the composition and dynamics of fungal communities, partly because many species cannot be cultivated outside their hosts. In collaboration with Nathaniel Street and Nicolas Delhomme at Umeå University, and other groups, we conducted a meta-transcriptome study of the spruce's endophytes based on RNA-Sequencing data generated from 22 tree samples, including 12 samples following the development from buds to two-year old needles, and needles infected by a fungal pathogen. While plant gene expression clearly groups by tissue, with wood and wood-like tissues on one end, and needles and their developmental stages on the other, fungal genes exhibit very sample specific expression patterns. Reconstruction of the mitochondrial small sub-unit (mtSSU) sequences and building phylogeny places 5 sequences with Capnodiales, and one with Pleosporales, both Dothideomycetes that have been shown to be endophytic and can take over critical roles for their hosts. As pathogen candidate, we identify one Rhytismatales, order Leotiomycetes, and another one not firmly phylogenetically placeable. In addition, we found 11 sequences that form their own clade outside of known fungi, constituting unknown or un-sequenced fungi. Our study shows that the spruce is host to a very diverse fungal community that is highly dynamic both with regards to abundance as well as composition.

HOST-RETROVIRUS EVOLUTION

Patric Jern

Retroviruses have challenged vertebrates for millions of years, occasionally leading to germ line integration and inheritance as endogenous proviruses, genetic parasites that provide unique opportunities to study the biology and evolution of host-virus relationships. Our research program involves studying retrovirus evolution and the activity and evolutionary contributions from inherited proviruses on host genome function. We are currently engaged in several projects to characterize endogenous retroviruses (ERVs), which constitute significant fractions of the hosts' DNA, in re-sequenced domestic animal genomes and in vertebrate genome assemblies. The known breeding history and well-studied phenotypic traits of domestic animals provide unique possibilities to study and establish connections among ERVs, chromosomal genes, phenotypes and biological function. To further our understanding of retrovirus evolution and the effects of retroviruses on host genome function, our main questions include: **(1)** How dynamic are ERV integrations in the host genome? **(2)** How much have ERVs, as a model for evolution, contributed to host genome function? **(3)** Do polymorphic ERVs contribute to phenotypic diversity? **(4)** What can the ERV fossil record uncover from evolutionary host-pathogen arms races that might apply to infectious retroviruses?

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Ahmed Arslan, (Masters degree project):
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Shahina Hayat, (Masters degree project):
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RETROVIRUS AND TRANSPOSABLE ELEMENT EVOLUTION

Alexander Hayward, Patric Jern

The growing catalogue of re-sequenced genomes and reference assemblies permits detailed comparative studies across the genomes of diverse organisms. We take advantage of this to characterize ERVs and DNA transposons in order to identify novel broad-scale patterns and processes of evolutionary importance. Specifically, we seek to elucidate the contributions that retroviruses and transposable genetic elements have had on the phenotypic evolution of their hosts. To this end, we combine a phylogenetic approach to construct evolutionary hypotheses of relationship with bioinformatics methodology. Since genetic divergence is often great among infectious retroviruses, ERVs or DNA transposons, an additional part of our research concerns developing improved means of extracting informative phylogenetic signal from these sequences.

COMPARATIVE GENOMICS AND GENETICS

Kerstin Lindblad-Toh

The overall research focus is on identification of disease genes and mutations of relevance for canine and human disease. Once genes, mutations and biological pathway causing disease have been identified, these can be used to develop better diagnostics and treatment options. Two major areas of research are utilized together to accomplish this; i) all the functional elements in the human and mammalian genomes need to be identified to understand what the functional mutations may be, ii) domestic animals allow us to more easily find disease mutations, genes and pathways and then translate these findings to human medicine.

The comparative genomics work is part of an ongoing collaboration with my group and other colleagues at the Broad Institute to find functional elements in the human genome and that of model organisms. This includes analysis of large numbers mammalian genomes to identify common constraint elements, of which two-thirds fall outside coding genes, and contain other functional signatures such as non-coding RNAs and associated RNA structures, potential enhancers and insulators. In addition, RNA-Seq analysis of many mammals and vertebrates is underway to get a more complete picture of the coding and non-coding transcriptome in these organisms. We also study genome evolution across vertebrates to understand how genomes change and allow organisms to adapt to novel environments. This year we published an important paper on stickleback evolution that showed that their adaptation to a freshwater environment happens in multiple locations using the same mutations and that many of these mutations are non-coding in nature. Substantial efforts have also gone into understanding the genomic signatures that accompanied dog domestication, where changes to genes underlying brain development and function as well as starch and fatty acid metabolism have been of importance.

The unique breeding history of the domestic dog offers an unparalleled opportunity to map genes important in disease susceptibility, morphology, and behaviour. The breed structures where certain genetic risk factors have been enriched within specific populations and where recent bottlenecks have generated long haplotypes makes the dog excellent for trait mapping. The dog is also a unique animal to use for comparative analysis since; dogs spontaneously get diseases with the same etiology as humans, they share largely the same environment and have roughly the same gene content. The past years our group has worked actively to map genes for both monogenic and complex traits including Amyotrophic Lateral Sclerosis, Obsessive Compulsive Disorder and Cardiomyopathy and Systemic Lupus Erythematous (SLE) like syndrome, an Auto Inflammatory Disease as well as multiple cancers. Currently identified mutations show a spectrum of variation types from point mutations and deletions within coding regions to regulatory insertions and duplications. For several other diseases we have identified strong candidate loci and are in the process of performing targeted resequencing using an in-house adapted sequence capture method followed by next generation sequencing. Interestingly for each complex trait a majority of candidate genes fall within specific pathway(s). For example, four of the candidate genes for the SLE like syndrome are all involved in T-cell activation through the NF-AT pathway. To follow-up on these results we are now also examining the same genes and pathways in human patients with the corresponding diseases.

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Publications 2010 to 2012

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CANCER

Malin Melin, Emma Ivansson, Maja Arendt, Ingegerd Elvers, Katarina Truvé (SLU)

Cancer is one of the most prevalent diseases in both humans and dogs and specific breeds often show a predilection for certain tumour types. The molecular basis of the increased cancer risk in the breeds is mostly unexplained and knowledge about the susceptibility genes may enable improved diagnosis and treatment. We have focused on identifying predisposing genetic risk factors for a selection of tumour types, including:

- Mammary tumours (breast cancer)
- Osteosarcoma (bone cancer)
- Lymphoma
- Mast cell tumours
- Glioma

A few high-risk breeds have been chosen for initial investigations of each tumour type. We have collected large case-control materials both in Europe and the US by extensive collaborations and have performed genome-wide association studies (GWAS) in a few hundred dogs per tumour type and breed using 170,000 SNPs. For each tumour type we identify multiple loci significantly associated with tumour development. Targeted resequencing has revealed a large number of candidate mutations that are currently being

validated and assessed for functionality. We are also performing tumour-normal and mRNA sequencing to identify somatic and expression level alterations in the canine tumours. By our analyses we have identified a large number of candidate genes for canine cancer and the genes are now being investigated further in dogs and human cancer patients. By translating the results to human cancer, this could provide a unique opportunity to improve diagnosis and treatment of cancer in both dogs and humans.

AUTOIMMUNE AND INFLAMMATORY DISEASES

SYSTEMIC LUPUS ERYTHEMATOSUS

Sergey Kozyrev, Fabiana Farias and Maria Wilbe (SLU)

The aim of this project is to use dog as a model for identification of new genes and gene networks behind the human autoimmune disease Systemic Lupus Erythematosus (SLE). SLE is a complex autoimmune disorder characterized by dysregulation of the immune system, which results in production of autoantibodies, generation of toxic autoantibody-antigen immune complexes (ICs), increased rate of apoptosis, defective clearance and complement activation. This leads to persistent inflammation and damage of peripheral organs and tissues. The human disease is more frequent in women of child-bearing age than in men (9:1). SLE has complex multigenic inheritance, and both canine and human diseases resemble each other in many clinical aspects. We have identified five loci associated with SLE in Nova Scotia duck tolling retrievers. Resequencing to identify candidate mutations and functional analysis of candidate genes and mutations is ongoing. Several genes located in the associated regions code for proteins involved in the NFAT signaling pathway acting downstream of T cell receptors, but well-known human SLE genes such as BANK1 are also found in the associated regions. In collaboration with Lars Rönnblom and Juha Kere we are also studying hundreds of genes in the pathways found in the canine study in a large cohort of human SLE patients, where phenotypic sub-classification is available. Our aim is to provide a molecular classification of patients based on affected pathways.

CANINE AND HUMAN AUTOINFLAMMATORY DISEASE

Mia Olsson, Jennifer Meadows

Autoinflammatory disease (AID) is defined as a dysregulation of the innate immune system, presenting clinically as unprovoked inflammation in the absence of high titre autoantibodies or antigen specific T cells. AID covers a spectrum of monogeneic and mulitfactorial diseases, with shared symptoms making diagnosis and treatment challenging. Whilst these diseases are rare in human populations, ~80% of patients are currently without known genetic cause. We use the Shar-Pei dog, a breed susceptible to homologous AIDs, as a comparative model for disease. In 2011 we showed that a genomic duplication upstream of the hyaluronan (HA) synthesizing gene, *HAS2*, was significantly associated with a periodic fever in these dogs, and proposed that the dysregulation of HA production could also play an important role in human health.

During 2012 we completed a second genetic analysis of Shar-Pei AID, SPAID. This time we used the careful phenotyping of many hundreds of individuals to expand the disease definition to include multiple signatures of inflammation; recurrent fever, arthritis,

breed specific secondary dermatitis, otitis, systemic reactive amyloidosis, and revealed that common shared risk loci were underlying each symptom. We continued the validation process for a genetic test for SPAID using new digital PCR technology to refine copy number estimates of the breed specific duplication. Lastly, in an international collaboration with both geneticists and clinicians, we have begun examining HA dysregulation in a variety of human AIDs, and have successfully implicated this glycosaminoglycan in previously genetically uncharacterised disease.

ATOPY AND ADDISON

Katarina Tengvall, Fabiana Farias, Marcin Kiercak (SLU), Jeanette Hansson (SLU)

Canine Atopic Dermatitis (CAD) is defined as a genetically predisposed allergic skin disease. The characteristic clinical features are most commonly associated with IgE antibodies directed towards environmental allergens. Typical signs of CAD are pruritus of the face, ears, paws, extremities, and ventrum. We have performed a GWAS of ~200 German shepherds. In addition, serum IgA was measured in the same individuals and was included in the GWAS as a covariate (low IgA compared to high IgA) to the CAD phenotype. These analyses generated two top candidate regions that are now being further investigated in dogs and human patients.

Addison's disease is an organ-specific disease and is generally caused by an immune-mediated destruction of the adrenal cortex tissue leading to glucocorticosteroid and mineralcorticoid deficiencies. Autoimmunity occurs when the central immunological tolerance is broken and the immune system fails to recognise its own tissue as self. The diagnosis of Addison's disease is diagnosed routinely by an ACTH stimulation test where artificial ACTH is injected and the cortisol levels in the sera are measured before and after the injection. We aim to identify the genetic risk factors in the high-risk breeds Standard Poodles, Bearded collies and Portuguese Waterdogs. Whole genome association mapping has been conducted in Swedish and US Standard Poodles. Analysis and additional phenotypic characterisation of cases and controls is ongoing.

METABOLIC AND CARDIOVASCULAR DISEASE

DIABETES

Maja Arendt, Abhi Ratnakumar, Tove Fall (SLU)

Hormone induced diabetes can develop in female dogs in relation to estrous or pregnancy. This is similar to diabetes developing in pregnant women. Certain dog breeds have a relatively high incidence of hormone related diabetes compared to others, indicating a genetic predisposition. We have performed genome wide associations studies comparing healthy and diseased dogs in two high risk dog breeds, the Border Collie and the Swedish Elkhound in order to identify regions in the genome associated with disease risk. In addition we have also looked for regions with reduced homozygocity within each breed to find genetic patterns, which are nearly or partly fixed within each breed. In the long term we are aiming to identify disease-associated genes leading to better understanding of the diabetes in general as well as improved manors of treatment and prevention which could benefit both human as well as dogs.

DILATED CARDIOMYOPATHY (DCM)

Jennifer Meadows, Susanne Björnerfeldt (SLU)

To date, at least 19 genes have been implicated in familial forms of human dilated cardiomyopathy (DCM). The majority of these have been shown to encode structural proteins essential to the heart muscle's contractile strength, however this still leaves many hereditary and idiopathic cases of disease without known genetic cause. There are several large- and giant dog breeds, which are also predisposed to DCM and the current study considered both purebred Great Danes (GD) and Newfoundlands (NF). The disease in these breeds is described histopathologically as the attenuated wavy fibre type, in which the myocytes appear thinner than normal and are separated by oedematous fluid. As opposed to the other form of canine DCM, this fluid space is generally free from fatty cell infiltrates.

In 2012, emphasis was placed on recruiting more strictly classified cases and controls for genome wide association analyses (GWAS) and on following up the health status of dogs previously recruited into the study. For the GDs, this meant 182 individuals from eight countries were available for further study and for the NFs the result was 133 individuals from two countries. This has added power to the GWAS allowing associate regions to be refined. A second round of targeted genome sequencing was also performed for each breed to increase the depth of coverage across eight previously identified candidate loci, four for GD and NF respectively. Genes of interest from each cardiac project will be carried forward into a human targeted sequencing program.

ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY (ARCV)

Jennifer Meadows, Susanne Björnerfeldt (SLU)

Arrhythmogenic right ventricular cardiomyopathy (ARVC) presents as arrhythmias and fibro-fatty replacement of the right ventricular myocardium. It is typically known as a disease of the desmosome, the structural hinge required for both myocardial strength and cell signaling, as this is where the majority of gene mutants linked to human disease are acting. A previous publication using approximately fifty thousand SNP typed across purebred boxer dogs from the US, identified a mutation in the 3' UTR of the gene striatin (*STRN*) as causal for (ARCV), but also suggested potential modifying loci as identified through genome wide association analyses (GWAS).
In 2012 targeted genome sequencing the four major peaks identified in the original canine GWAS was undertaken using individuals from that study. In addition, was integrated with samples from extremely well phenotyped purebred boxers from the United Kingdom, which had been previously shown not to segregate the STRN mutation. That experiment will use GWAS and the data from the Illumina 170K SNP array for 101 purebred boxer dogs from the UK to investigate ARVC in that population.

NEUROLOGICAL AND BEHAVIOURAL DISEASE

DEGENERATIVE MYELOPATHY

Emma Ivansson

Canine degenerative myelopathy (DM) is a severe neurodegenerative disease of late onset. Signature features of the disease are axonal and myelin degeneration of the spinal cord causing progressive ataxia and paresis. We have mapped and identified a mutation in the *SOD1* gene as the major cause of DM in at least five dog breeds using genome-wide association analysis (GWAs) in the Pembroke Welsh Corgi breed and further validation in other breeds. A mutation in human *SOD1* causes a similar neurodegenerative disease, amyotrophic lateral sclerosis (ALS), suggesting that that this is a good animal model for human ALS. The identified *SOD1* E40K missense mutation appears to encode a major risk factor for the disease but is not fully penetrant, suggesting that additional modifier loci may be involved. In humans the identified *SOD1* mutations account for only 20% of the familial ALS also suggesting other genetic variations to be involved in disease development. We have performed additional GWAs and resequencing in carriers of the SOD1 risk allele to identify genetic modifiers that can predict disease severity and onset as well as identifying other genetic variations that can cause disease. We are also collaborating with Ingela Nyström at Akademiska Hospital to follow up genes and pathways identified in the canine breeds with targeted resequencing of human ALS patients.

INVESTIGATING GENETICS UNDERLYING BEHAVIORAL TRAITS IN DOGS

Marcin Kiercak (SLU), Katarina Tengvall and Fabiana Farias

Dogs have often been bred for specific behavioral traits. During dog domestication traits important for interactions with people was selected. Later specific behaviors have been selection for specific breeds. Examples include herding, hunting or friendliness. Since 1989 the Swedish Working Dog Association has been carrying Dog Mentality Assessment (DMA or MH in Swedish) test, which consists of several standardized situations, e.g. exposure to sudden metallic noise. The behavior of all tested dog are evaluated by a professional judge and several traits are measured, e.g. intensity of social contact, playfulness, eagerness to chase.

In our study, we have initially focused on German shepherds (GSD) which are often used as working dogs and that also show certain within-breed variation in behaviour. Our pilot study, based on more than a hundred genotyped GSDs, revealed several regions in the genome that show interesting associations with behaviours such as: chasing, aggression, curiosity, playfulness and sociability. This dataset is now being complemented by a number of carefully-selected shepherds that represent the whole spectrum of observed behaviours. Apart from GSDs, we are analysing data from other breeds to understand breed-specific behaviours.

While the studied traits describe normal variation within dog breeds, a number of human behavioural diagnoses such as anxiety, autism, ADHD or depression may be caused by similar mechanisms. Thus, our study can potentially contribute to the development of diagnostic tools and novel treatments from which also humans will benefit.

EVOLUTION

GENETIC AND FUNCTIONAL CHARACTERISATION OF DOG DOMESTICATION.

Erik Axelsson, Abhi Ratnakumar, Maja Arendt

The dog may have been the first animal to be domesticated and has since been an integral part of human culture. Up until recently our understanding of the genetic basis of traits separating the dog from its wild ancestor the wolf was limited. Our group has now completed a survey of genetic diversity in dog and wolf using light-coverage whole genome resequencing. This data has allowed us to shed new light on the molecular basis of early dog domestication by identifying and functionally characterising genomic regions that were selected for during the initial, pre-breed, phase of the domesticating process. Our results show that (1) dogs have adapted to cope with a starch-rich diet and (2) that selection during dog domestication affected mutations in nervous system development genes - changes that are likely to underlie several of the behavioural differences between dogs and wolves. We now plan to build on this work in three ways.

First, by screening both contemporary, and ancient, dog and wolf populations for mutations that alter the efficiency of starch digestion we aim at determining how variable this trait is among dog breeds, whether it is associated with metabolic disorders and when selection for this trait may have started.

Secondly, by testing if candidate mutations that affect nervous system development genes have led to functional differences between dog and wolf we aim at identifying mutations that underlie behavioural changes during dog domestication.

Finally, to further increase our understanding of the molecular basis of dog domestication we will extend our survey of genetic diversity in dog and wolf populations by additional whole genome resequencing.

COMPARATIVE GENETICS OF IMMUNOLOGICAL DISEASES TOWARDS FUNCTIONAL GENOMICS

Gerli Rosengren Pielberg

The overall aim of the research group is to take advantage of information from comparative genetics and provide functional genomics data both in model organisms as well as humans. We are working with dog as a model organism and more precisely breeds predisposed to immunological and immune-mediated diseases with comparative value to human diseases.

Dogs, as other domestic animals, have a genomic structure suitable for gene mapping. In addition, dogs also present the same spectrum of diseases as humans and nowadays even share our living-environment. Therefore, taking advantage of those characteristics may provide us knowledge of genetic risk factors lying behind human diseases. In general our approach goes from identifying risk loci in a dog breed all the way to providing functional evidence of an identified mutation contributing to the disease development. Our goal is to provide information necessary for the future development of genetic testing, diagnostics and therapy for the dogs. Furthermore, the ultimate goal is to provide functional genomic data of the same mutations, genes and pathways being involved in the development of human homologous diseases.

Members of the group during 2012
Gerli Rosengren Pielberg, Assistant Professor, group leader
Matteo Bianchi, PhD student

Project worker during 2012
Hannes Hällgren, SoFoSko student

Publications 2010 to 2012

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CHARACTERIZATION OF GENETIC RISK FACTORS BEHIND LYMPHOCYTIC THYROIDITIS

Matteo Bianchi, Gerli Rosengren Pielberg

Lymphocytic Thyroiditis is one of the most frequent endocrinopathies in dogs, affecting multiple breeds. The disease is characterized by autoimmune destruction of the thyroid gland resulting in functional failure of the thyroid. The homologous disease in humans is called Hashimoto's Thyroiditis, resembling most clinical aspects of the disease in dogs.

The overall aim of this project is to use dog as a model organism to identify mutations, genes, and pathways behind development of human Thyroiditis. We have performed a genome-wide association analysis and identified several candidate loci in different dog breeds. We have also identified hundreds of potential candidate mutations by targeted resequencing of candidate loci. Currently we are in the process of high-throughput genotyping of potential candidate mutations as well as functional validation of obvious candidate mutations. In the future we are planning to screen human Thyroiditis patients for mutations in genes and pathways identified as involved in the development of the canine disease.

The results from this study may lead to development of genetic tests and better diagnostic methods as well as new alternative therapies for treatment of both canine and human patients.

GENOME EVOLUTION

Matthew T Webster

We study evolution on the molecular level by comparing genomes of different species and analysing patterns of genetic variation on the whole-genome scale, using bioinformatic and statistical approaches. We are interested how molecular forces such as meiotic recombination interact with natural and artificial selection to shape the evolution of genomes and the phenotypes they produce.

We are currently using massively-parallel sequencing to characterise global patterns of genetic variation in the honeybee. A major goal of this project is to identify genes important for adaptation, which could be vital to protect this important species from colony losses. Using the domestic dog as a model, we are performing a whole-genome analysis of recombination hotspots and copy number variation, in order to understand the molecular mechanisms that lead to genome instability. We have performed an analysis of molecular footprints of artificial selection in the dog genome, and are currently attempting to characterise mutations with important functional effects for dog domestication.

Members of the group during 2012
Matthew Webster, group leader
Andreas Wallberg, postdoc
Abhi Ratnakumar, PhD student
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Gustaf Wellhagen (bioinformatics masters student, UU)

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MOLECULAR BASIS OF ADAPTATION IN THE HONEYBEE, *APIS MELLIFERA*

Andreas Wallberg, Fan Han, Gustaf Wellhagen

The honeybee is vital for maintaining levels of biodiversity and agricultural production through its role in plant pollination. However, it is threatened by several factors, including pathogens, biological invasions, climate change and pollution. Honeybees, and the plants that rely on them, are in decline, incurring major ecological and economic costs. Honeybees are grouped into a number of subspecies, which are estimated to have diverged and spread across Africa and Eurasia around one million years ago. Natural selection resulted in each of these subspecies becoming adapted to its local environment. More recently, the management of colonies by humans has resulted in artificial selection for desirable traits.

Our goal is to uncover the molecular basis of these traits. These include traits common in certain races, such as cold adaptation and gentleness. In addition, certain traits, such as parasite resistance and hygienic behaviour are important for honeybee health and viability. We have sampled populations drawn from several honeybee subspecies, and from populations of honeybees specifically selected for disease resistance and are surveying genetic variation across the entire genome in these populations using next-generation sequencing. We then analyse these fine-scale patterns of genetic variation for the characteristic footprints of "selective sweeps" which indicate genes or genomic regions that are responsible variation in traits of interest.

RECOMBINATION AND GENOME EVOLUTION

Jonas Berglund

Meiotic recombination is a fundamental biological process, which maintains genetic variation within populations and is essential for chromosomal segregation. However, recombination may also have damaging effects on the genome, either as a cause of structural mutations or due to a process known as biased gene conversion, which alters the frequency of mutations in a population. The genomic distribution of recombination events is localized to specialized sites known as hotspots, but the mechanisms controlling this variability are unclear.

The dog genome is unusual among mammals in that it lacks a key protein that initiates recombination in other species. We have used genomewide genetic variation data to map the locations of recombination hotstpots in dogs and uncovered unique features that may reflect novel mechanisms of the control of recombination. We are now using comparative genomic approaches to investigate how these mechanisms might affect the human genome. Furthermore, we are analysing array comparative genomic hybridisation data from various dog breeds in order to identify copy number variants and understand the role of recombinogenic mechanisms in their generation.

GENOME SCANS FOR SELECTION IN DOGS

Abhi Ratnakumar

There are hundreds of dog breeds that exhibit massive differences in appearance and behaviour sculpted by tightly controlled selective breeding. This large-scale natural experiment has provided an ideal resource that geneticists can use to search for genetic variants that control these differences. In collaboration with Illumina and the LUPA consortium for dog disease mapping, we have developed a high-density genotyping array (canineHD) that surveys variable sites at more than 170,000 positions in the dog genome. This array has been now been used to analyse genetic variation in thousands of dogs. We have analysed these data to identify chromosomal regions that are extremely variable between breeds and are likely to control many of the traits that vary between them. We are characterising one such region in more detail, which associates with differences in body size and ear type between breeds. We are using “next-generation” sequencing technology to identify candidate mutations that may control these traits. Our results suggest that artificial selection has targeted genes involved in development and metabolism and that it may have increased the incidence of disease in dog breeds. Knowledge of these regions will be of great importance for uncovering the genetic basis of variation between dog breeds and for finding mutations that cause disease.

MEDICAL BIOCHEMISTRY

GLYCOBIOLOGY

PROTEOGLYCANS - BIOSYNTHESIS AND BIOLOGICAL FUNCTIONS

Cecilia Annerén, Lena Kjellén, Johan Kreuger, Jin-ping Li, Ulf Lindahl, Maria Ringvall, Dorothe Spillmann

The IMBIM groups active in this area study proteoglycans and elucidate functional aspects of these glycoconjugates in relation to embryonic development, angiogenesis and during pathophysiological conditions such as amyloidosis, inflammation and tumor progression. In addition, mouse and human embryonic stem cells are studied focusing on molecular mechanisms that regulate self-renewal as well as roles of proteoglycans in differentiation into different lineages. Many collaborations between the groups create a strong unit.

A majority of the projects concerns heparan sulfate proteoglycans. Heparan sulfate modulates growth factor and cytokine action and participates in the generation and maintenance of morphogen gradients and is therefore of particular importance both during embryonic development and in different pathologies. Biosynthesis of heparan sulfate and its regulation is one important focus. Recent projects also address the question of functional overlaps between heparan sulfate and chondroitin sulfate proteoglycans. Model systems include mice and zebrafish and most recently C. elegans.

MOLECULAR MECHANISMS REGULATING PLURIPOTENCY AND SELF-RENEWAL OF PLURIPOTENT STEM CELLS

Cecilia Annerén

Pluripotent stem (PS) cells, i.e. embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have the ability to maintain pluripotency during long-term culture and yet induce differentiation into multiple lineages and thus potentially offers novel cell sources for toxicological screening, *in vitro* modelling of genetic disorders or therapeutic cell replacement. However, before these endpoints can be fully realized, it will be necessary to find defined culture conditions that support simple and robust large-scale expansion protocols, while maintaining self-renewal, of these difficult to culture cells. Moreover, specialized cells obtained from differentiated PS cultures may contain a small amount of undifferentiated PS cells with tumorigenic properties and there is therefore important to recognize and control the signalling pathways regulating PS cell self-renewal, proliferation and pluripotency if these are to be used in a clinical setting. For all the reasons above, a very important and challenging issue in stem cell biology is to understand the mechanisms that regulate self-renewal, including the maintenance of growth, survival and the undifferentiated state, of mouse and human PS cells as well as developing robust and validated technologies for handling these cells.

Human and mouse PS cells respond differently to various growth factors or cytokines. Whereas at least some mouse ES (mES) cell lines can self-renew on gelatin in the presence of Leukemia inhibitory factor (LIF); human ES (HUES) cells are usually cultured on feeder cells or matrigel in media containing Fibroblast growth factor (FGF)2/bFGF. LIF maintains self-renewal of mES cells and activates various pathways, including the JAK-STAT3, PI3K-AKT, MAPK and cYes-YAP-TEAD2 cascades, leading to the expression of several transcription factors such as Oct3/4 and Nanog, known to be essential for mouse PS cell self-renewal. LIF is, however, not able to sustain self-renewal of human PS cells. Despite that there are significant species differences, some critical and conserved pathways and transcriptional regulators are conserved in mouse and human PS cells, such as the core transcriptional proteins Oct3/4, Sox2 and Nanog.

Our goal is to gain a better knowledge of individual genes in the context of self-renewal in human and mouse PS cells. We also want to directly compare various standard culture conditions and newly developed ones with the aim of developing new cell culture media, reagents, surfaces or applications for large-scale expansion of human PS cells. We anticipate that the knowledge obtained from this work, will prove to be invaluable in future work on mouse and human PS cells, by providing a deeper molecular understanding of the *in vitro* propagation and differentiation of these cells as well as the use of human PS cells in tissue regeneration and transplantation.

Members of the group
Cecilia Annerén, Ph.D., Adjunct Senior Lecturer
Christoffer Tamm (Post doc)
Sara Pijuan Galitó (Ph.D. student)

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Agencies that support our work

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STUDY OF DIFFERENT PROTOCOLS FOR MOUSE EMBRYONIC STEM CELL CULTURE

Christoffer Tamm, Sara Pijuan Galitó

Most stem cell laboratories still rely on old culture methods to support the expansion and maintenance of mouse embryonic stem (ES) cells. These involve growing cells on mouse embryonic fibroblast feeder cells or on gelatin in media supplemented with fetal bovine serum and leukemia inhibitory factor (LIF). However, these techniques have several drawbacks including the need for feeder-cells (for feeder-dependent cell lines) and use of undefined media containing animal derived components. Culture of stem cells under undefined conditions can induce spontaneous differentiation and reduce reproducibility of experiments. In recent years several new ES cell culture protocols, using more well-defined conditions, have been published and we are presently comparing the standard culture protocols with the newly described ones: e.g. the growing of cells in semi-adherence in a medium containing two small molecule inhibitors (CHIR99021, PD0325901) and the growing of cells in a spheroid suspension culture in a defined medium containing LIF and bFGF. The overall aim is not only been to compare self-renewal and differentiation capacity, but also ease-of-use and cost efficiency. We can see that mES cells when grown adherently proliferate much faster than when grown in suspension as free-floating spheres, independent of media used. Although all the tested culture protocols maintain sustained pluripotency after prolonged culturing, we can confirm previous reports showing that the media containing two chemical inhibitors generate more pure stem cell cultures with negligible signs of spontaneous differentiation as compared to standard mES media. Furthermore, we see that this medium effectively rescue and clean up cultures that have started to deteriorate, as well as allow for effective adaption of feeder-dependent mES cell lines to be maintained in feeder-free conditions.

STUDY OF DIFFERENT PROTOCOLS FOR HUMAN EMBRYONIC STEM CELL CULTURE AND TRANSFECTION

Sara Pijuan Galitó, Christoffer Tamm

Human PS cells, i.e. human embryonic stem (HUES) cells and iPS cells are traditionally cultured on feeder layers or Matrigel in FGF2-containing media. Passaging of these cells is performed by manual cutting techniques or dissociating the cells in clumps using a very gentle dissociating method. Very recently a number of studies have been published on microcarrier culture of human PS cells and the development of new synthetic surfaces for human ES cells growth. Moreover, several novel defined media have recently been launched on the market. We are currently benchmarking commercially available and novel products for PS cell culture. A long-term goal is to be able to use these techniques for industrial scale expansion of stem cells. Another aim is to develop better methods for generating engineered PS cells for research or clinical purposes. So far we have obtained some interesting results when introducing small interfering RNA using a novel self-penetrating peptide in HUES and iPS cells.

ROLE OF A NOVEL SERUM PROTEIN AND THE CYES/YAP/TEAD2 PATHWAY FOR SELF-RENEWAL AND ATTACHMENT OF MOUSE ES CELLS

Sara Pijuan Galitó, Christoffer Tamm

We have previously shown that the cytoplasmic tyrosine kinase cYes is activated by LIF and play an important role in maintaining mES cell self-renewal (Annerén et al. 2004). We recently identified TEAD2 and the Yes-associated protein YAP; its transcriptional co-activator, as co-operating in a signalling pathway downstream of LIF, and cYes (Tamm et al, 2010). Moreover, we have observed that the Yes/YAP/TEAD2 pathway is, in addition to LIF, activated by fetal bovine serum (FBS) in a time and dose-dependent manner (unpublished results). It is known that both LIF and serum are important for self-renewal and maintenance of pluripotency of the mES cells. The serum factor BMP4 has been shown to be able to replace FBS in mES cell cultures but BMP4 cannot activate the cYes/YAP/TEAD2 pathway, suggesting that other factor(s) present in serum is responsible for this effect. By fractionation of serum, using different purification/separation techniques e.g. different types of chromatography, precipitation and filtration, we have managed to isolate and identify a serum protein (SP) as the likely candidate for activating this pathway (unpublished results). So far we have found that the purified SP can activate TEAD2-dependent transcription in a dose dependent manner and that SP, similar to LIF, induce YAP nuclear translocation (YAP is a co-trancription factor that, when activated, is translocated to the nucleus bind TEAD2 and activates TEAD2 dependent transcription). We have also found that addition of SP promotes ES cell attachment under serum-free media conditions. The exact mode of action for SP-induced TEAD2 dependent transcription is yet to be shown. To further discern the effect of the SP and to identify its putative signal transduction pathways, we are currently in collaboration with GE Healthcare performing a proteomics analysis using *2-dimensional Difference Gel Electrophoresis (2D-DIGE)* assay.

CELLULAR DESIGN OF HEPARAN SULFATE

Lena Kjellén

Heparan sulfate structure varies greatly during embryonic development and differs also when heparan sulfate isolated from different tissues and cell types of an adult animal are compared. Biosynthesis takes place in the Golgi compartment and relies on the action of a multitude of enzymes. Our main goals are to find out how the cell decides on a particular heparan sulfate design and to characterize the molecular machinery responsible for its biosynthesis. Our model systems are mouse and zebrafish where we study study biological effects of mutations in biosynthesis enzymes. Embryonic stem cells and embryonic fibroblasts derived from mutant mice as well as mammalian cell-lines overexpressing or lacking selected biosynthesis enzymes are important tools. A sensitive method to determine glycosaminoglycan concentration and structure is available in the lab, enabling analysis of cultured cells as well as small tissue samples. Our focus has been on the biosynthesis enzyme glucosaminyl N-deacetylase/N-sulfotransferase, NDST, which has a key role in heparan sulfate design during biosynthesis in the Golgi compartment. NDST removes acetyl groups from glucosamine residues and replaces them with sulfate groups. These N-sulfate groups are important for further modifications including O-sulfation in various positions and epimerization of glucuronic acid to iduronic acid. Four NDST isoforms, transcribed from four genes, have been identified.

Sulfation, obviously important in heparan sulfate biosynthesis, also regulates protein and steroid hormone action. New projects in the lab include characterization of sulfate metabolism in zebrafish and in cancer metastasis. Heparan sulfate biosynthesis in mucopolysaccharidoses is also a new area studied in the group.

Members of the group during 2012
Anders Dagälv, postdoc
Audrey Deligny, postdoc
Tabea Dierker, postdoc
Anh-Tri Do, postdoc
Inger Eriksson, research engineer
Beata Filipek-Górniok, graduate student
Lena Kjellén, professor, group leader
Dagmar Sandbäck Pikas, researcher

Project workers during 2012
Magnus Rosling, Sofosko student

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*equal contribution
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REGULATION OF HEPARAN SULFATE BIOSYNTHESIS/ IN SEARCH FOR THE GAGOSOME

Audrey Deligny, Dagmar Sandbäck Pikas, Inger Eriksson

Our previous results support a GAGosome model where biosynthesis enzymes are assembled into modifying units and the composition of the unit determines the outcome of

biosynthesis. This model is now being challenged and potential interactions between biosynthesis enzymes are being explored. Our recent finding of altered heparan sulfate biosynthesis in Hurler syndrome will be the basis for a more general characterization of this process in other mucopolysaccharidoses, a group of lysosomal storage diseases caused by mutations in glycosaminoglycan degradative enzymes.

MAST CELL PROTEOGLYCANS

Anders Dagälv, Inger Eriksson

Previously, serglycin was the only proteoglycan characterized in mast cells. This proteoglycan is found inside the cells in the granulae where it is essential for the storage of inflammatory mediators. Our preliminary results indicate that mast cells also synthesize cell surface proteoglycans. We are now characterizing these proteoglycans with regard to core protein identity and structural fetures of their heparan sulfate chains. Mice with targeted mutations in the core protins identified will be used to study functional aspects of the cell surface proteoglycans.
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HEPARAN SULFATE BIOSYNTHESIS AND METABOLISM OF THE SULFATE DONOR PAPS IN ZEBRAFISH

Beata Filipek-Górniok

We are in collaboration with Johan Ledin, EBC, characterizing zebrafish NDSTs with regard to expression and isoform function. In addition, metabolism of the general sulfate donor PAPS is investigated and the impact of PAPS availability for glycosaminoglycan structure and function is studied

SULFATE METABOLISM IN CANCER

Anh-Tri Do, Magnus Rosling, Beata Filipek-Górniok

Altered PAPS metabolism in the context of cancer development and metastasis is a previously almost unexplored area. In addition to its important role in heparan and chondroitin sulfate biosynthesis, PAPS is also the donator of sulfate when sex hormones are sulfated, a substitution which results in inactivation of the hormones. Zebrafish will be used as a model to study the impact of PAPS concentration on invasion and metastasis of breast cancer and prostate cancer cell lines. Mutant fish with lowered PAPS concentration as well as cell lines where PAPS production has been manipulated will be used in the studies.

FUNCTIONAL OVERLAP BETWEEN HEPARAN SULFATE AND CHONDROITIN SULFATE

Tabea Dierker

Recent results suggest that chondroitin sulfate sometimes can substitute for heparan sulfate in physiologically important interactions. To understand and characterize this overlap is

particularly important in light of the increased interest of the development of glycosaminoglycan mimetics for therapeutic purposes. The nematode *C. elegans* synthesizes heparan sulfate which in all structural aspects is similar to the mammalian polysaccharide. In contrast, only chondroitin without any sulfate substitution is produced by the nematode. *C*. *elegans* mutants with defective heparan sulfate biosynthesis show a strong misrouting of motor axons. To investigate if chondroitin sulfate can substitute for heparan sulfate we will, in collaboration with Andrea Hinas at IMBIM, introduce different zebrafish chondroitin sulfate sulfotransferases into these mutants, confirm that the worm now is able to produce sulfated chondroitin and investigate how this affects the routing of the motor axons.

FUNCTIONAL STUDIES OF BLOOD VESSEL GUIDANCE

Johan Kreuger

The overall objective of our research team is to increase our understanding of how signaling gradients control formation of new blood vessels. Detailed information on how multiple instructive gradients together steer blood vessel growth is currently lacking.

The rationale for our research is that increased understanding of how gradients control angiogenesis ultimately will lead to new approaches to treat pathological angiogenesis, occurring for example in association with cancer.

The research activities in our group are cross-disciplinary. We use advanced cell culture systems, various disease models, novel biotech tools and biochemical approaches to study directional angiogenesis.

We further collaborate closely with Prof. Pär Gerwins at IMBIM/Uppsala University Hospital to identify mutations that cause vascular malformations. Within this disease group there are some rare conditions that cause substantial morbidity with severe pain, ulcerations, cardiac failure, amputations and death. Our studies aim at increasing our basic understanding of the molecular mechanisms behind these rare but severe diseases, with the ultimate goal of finding new pharmacological treatment strategies.

Members of the group
Johan Heldin, PhD student
Zsolt Kasza, Postdoc
Paul O'Callaghan, Postdoc
François Binet, Postdoc

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The Swedish Research Council
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Polysackaridforskning AB

Publications 2010 to 2012

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Patents 2010 to 2012

2010 Microfluidic capsule - SE 1050936-2
Describes a novel and multifunctional thermoplastic capsule, which enables straightforward operation of a multitude of microfluidic elastomer systems for life science applications.

VASCULAR DEVELOPMENT AND PATHFINDING IN RESPONSE TO GROWTH FACTOR GRADIENTS

Paul O'Callaghan, Johan Heldin, Zsolt Kasza

Directional migration of endothelial cells is important for angiogenesis in both normal development and during many diseases such as cancer, atherosclerosis and proliferative retinopathies. Our goal is to better understand the basic mechanisms behind endothelial cell chemotaxis. We have identified a set of genes, including the exocyst component *exoc3l2,* which is selectively expressed by growing blood vessel sprouts. We are now further characterizing the role of *exoc3l2* as well as a relatively uncharacterized guanine nucleotide exchange factor in angiogenesis.

PROTEOGLYCANS REGULATING TISSUE DEVELOPMENT

Zsolt Kasza, Paul O'Callaghan

Heparan sulfate proteoglycans (HSPGs) are critical for vascular development, with capacity to modulate and potentiate VEGF-receptor mediated angiogenesis. In addition, HS has been implicated in recruitment of pericytes, a special type of supporting cell, to small vessels by allowing formation of attractive PDGF gradients. We focus our study on the capacity of HSPGs to activate tyrosine kinase receptors to allow for development of pericytes and capillary structures from clusters of differentiating stem cells. We have also identified several genes and miRNAs selectively expressed at high levels by actively sprouting blood vessels, in order find gene regulatory networks that promote angiogenesis.

We are now knocking down genes and miRNAs in vascular cells to identify their roles in the formation, patterning and path finding of growing blood vessels.

IN SEARCH FOR GENETIC CAUSES OF PARKES WEBER SYNDROME- A SEVERE VASCULAR ANOMALY THAT IS DIFFICULT TO TREAT

Pär Gerwins (PI), Johan Kreuger, François Binet

Congenital malformations in different components of the vascular system give rise to conditions known as vascular anomalies. Within this group there are some rare conditions that cause severe pain, ulcerations, cardiac failure, amputations and substantial morbidity. The purpose of this project is to reveal mutations associated with Parkes Weber syndrome, a complex disorder of the vascular system. Whole-genome sequencing of a family of four individuals where the two children but not the parents have been diagnosed Parkes Weber will be performed. Today there are no effective treatments for Parkes Weber. This project represents the beginning of a larger effort to increase our basic understanding of the molecular mechanisms behind this rare but very severe disease, with the ultimate goal of finding new pharmacological treatment strategies.

HEPARAN SULFATE AND HEPARANASE: IMPLICATIONS TO ANIMAL DEVELOPMENT AND PATHOPHYSIOLOGICAL PROCESSES

Jin-ping Li

The research of this group aims at elucidating the functional properties of heparan sulfate (HS) in animal development and homeostasis, as well as under pathological conditions. To study the functions of HS in animal development, we have generated transgenic mice by interfering expression of genes involved in HS biosynthesis and modification. To study the effects of HS in pathological conditions, we apply these transgenic mice to different models of diseases such as amyloidosis, inflammation, atherosclerosis and cancer. We also collaborate with clinical researchers to correlate our findings from animal models with clinical diseases. We focus our investigation on the specific effects of two key enzymes involved in HS biosynthesis (glucuronyl C5-epimerase) and modification (heparanase) in the disease conditions. We use various techniques including biochemical, molecular and immunohistological tools as well as mouse models.

Members of the group during 2012
Mahsa Shahidi Dadras, Master student (until April)
Adreas Digre, graduate student
Jin-ping Li, MD, PhD, group leader
Ulf Lindahl, PhD, professor emeritus
Fredrik Noborn, graduate student (until May)
Ying-xia Tan, Post-doc (until November)
Wimal Ubhayasekera, Post-doc (until June)

Project worker during 2012
Erika Manlig, summer student (June-July)

International exchange during 2012
Visitors to my lab
Robert Kisilevsky (Canada), Prof. emeritus, one week in May
Xiaofang Cao, 3 months (Oct-Dec)

Group member to visit other lab
Jin-ping Li, visited Oncology Department of Beijing Hospital of traditional Chinese Medicine, one week in December

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HEPARAN SULFATE IN ANIMAL DEVELOPMENT

Jin-ping Li

Heparan sulfate (HS) is an essential molecule in animal development, as demonstrated by early embryonic lethality of mice defect in HS synthesis. Glucuronyl C5-epimerase, one of the enzymes involved in HS biosynthesis, catalyzes the conversion of D-glucuronic acid (GlcA) to its C5-epimer, L-iduronic acid (IdoA), at the HS polymer level. The IdoA units are believed to promote binding of HS chains to ligands, due to the marked conformational

flexibility of these residues. Therefore, the reaction catalyzed by the C5-epimerase is crucial for many biological functions of HS.

Targeted disruption of the GlcA C5-epimerase gene in mouse resulted in neonatal lethality of the animals, with a severely disturbed developmental phenotype, such as renal agenesis, lung hyperplasia and multiple skeletal malformations. Analysis of HS isolated from mutant animals revealed a perturbed structure completely lacking IdoA residues, but with increased *N*- and 6-*O*-sulfation contents. The alteration of the HS structure apparently affected functions of a number of cytokines, e.g. FGF-2, that require HS as co-receptors. Recently, we have found that the GlcA C5-epimerase is involved in lymph organ development (in collaboration with Prof. S. Pals, the Netherlands).

To continue the study, we are looking for collaborations with experts experienced in development of renal, lung and skeletal systems. The collaboration is open to researchers to join our group or at independent labs. Another ongoing approach is to generate a conditional GlcA C5-epimerase knockout mouse to enable selective inactivation of the gene in different organ or cells.

STURCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE ENZYMES INVOLVED IN HEPARAN SULFATE BIOSYNTHESIS

Wimal Ubhayasekera

Biosynthesis of HS is a complex process; the action of at least 11 different enzymes results in polysaccharide molecules with a high degree of heterogeneity. As the structure of HS is highly tissue/cell specific, our primary interest is to find out how the biosynthesis is regulated *de novo*. Approaches to understanding the organization of HS biosynthesis involve characterization of the "gagosome", *i.e.* the complex of (largely) membrane-bound proteins in the Golgi. Particular attention will be given to the interactions between enzymes, e.g. GlcA C5-epimerase and O-sulfotransferases. Recombinant enzymes (GlcA C5-epimerase, HexA 2-O-sulfotransferase and GlcN 6-O-sulfotransferase) are applied to modify polysaccharide substrates for investigation of: 1) substrate specificity of the individual enzymes; 2) interaction/regulation of the enzymes in their separate or concerted action towards various substrates; 3) kinetics of the enzymatic reactions. In more complex biological systems, tissues, cells or sub-cellular organelles (in particular Golgi fractions) derived from transgenic mice (GlcA C5-epimerase KO, heparanase KO and heparanase overexpression) will be used for identification of enzyme complexes (the "gagosome"), using various analytical approaches.

Another line of this project is to illustrate the molecular structures of the enzymes by X-ray crystallographic technique. The recombinant enzymes, especially GlcA-epimerase, are used for crystallization. Further plan is to co-crystallize the enzymes with oligosaccharide substrate to define the catalytic site through mutations of critical amino acid residues in the proteins.

STRUCTURE AND FUNCTIONS OF HEPARAN SULFATE IN AMYLOIDOSIS

Fredrik Noborn, Andreas Digre

"Amyloidosis" refers to a clinical condition encompassing a group of more than 20 post-secretory protein misfolding diseases. In these disease states, proteins that are normally soluble undergo aggregation to form insoluble fibrils and are accumulated in the extracellular space (also intracellular) of affected tissues or organs. A common feature of

all amyloidosis diseases is the selective organ deposition of disease-specific fibrillar proteins along with HS-proteoglycans (HSPGs). HS and HSPGs appear not to be merely passive components of amyloid deposits but rather play functional roles in the pathophysiology of amyloidosis. Two types of amyloid diseases that have a broad clinical and social impact are Alzheimer's disease (AD) and type 2 diabetes.

As HS is pertinently found in all types of amyloid deposits, it is of importance to find out the functions of HS in these diseases. We primarily focus the studies on few-selected amyloidosis, e.g. systemic amyloidosis caused by deposition of transthyretin (TTR) in the heart; inflammation associated amyloid A (SAA) deposition in the spleen/liver/kidney; type II diabetes (IAPP deposition in the pancreas) and Alzheimer's disease (Aß deposition in the brain). Approaches taken include: a) *in vitro* studies to investigate the effects of HS and heparin in aggregation of the amyloid peptides, with regard to HS/heparin chain length and sulfation pattern; b) cellular studies to find out the roles of cell surface HS for internalization and toxicity of the amyloid peptides; different cell models with distinct HS property are used; c) animal models to address the *in vivo* functional roles of HS in amyloidosis.

HEPARANASE – A MODULATOR IN BLOOD COAGULATION?

Ying-xia Tan

Heparanase is an endo-glucuronidase that cleaves HS and heparan. The enzyme was discovered first in a mast cell tumor in 1975, believed to be specific for cleavage of heparin. Later the same enzymatic activity was found in all organs. At normal conditions, the enzyme is expressed at a relatively low level in most of organs, essentially non-detectable by Western blotting technique. However, the enzyme is significantly upregulated at several pathological conditions, such as inflammation and cancers.

To study the functions of heparanase, we have generated transgenic mice, the heparanase-overexpression mice that overexpress human heparanase (Hpa-tg) and the heparanase knockout mouse (Hpa-KO) that is generated by targeted interruption of the gene. We have used these mice for investigation of different diseases by applying disease-specific models. Our earlier studies have revealed that heparanase modulates the activity of mast cells, through degradation of heparin that is a potent anticoagulant broadly used in clinic. To continue this study, we are looking at the effect of heparanase in blood coagulation, with focus on coagulation disorder related cardiovascular system diseases.

IMPLICATIONS OF HEPRANASE IN RHEUMATOID ATHRITIS

Andreas Digre

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by aggressive proliferation of synovial tissue (ST), leading to destruction of bone and cartilage in joints. Early changes in the synovia are characterized by revascularization, a marked infiltration of inflammatory cells, and associated synoviocyte hyperplasia, where angiogenesis is being recognized as an early event enabling activated monocytes to enter the synovia via endothelial cells.

Apart from the important functions of heparanase in angiogenesis, this enzyme is found upregulated under different types of inflammatory conditions. Particularly, it is

found dramatically increased (more than 100-fold) in the synovial fluid of rheumatoid arthritis (RA) patients. However, the underlying mechanisms are unknown. Our major question is: *what is the role of heparanase in RA pathology?* We will address this question by applying the collagen II-induced RA mouse model on our unique transgenic mice that are either overexpressing or lacking heparanase. In addition to the animal model, we will also examine the functions of synovial fibroblasts.

PROTEOGLYCANS IN REGULATION OF TUMOR ANGIOGENESIS AND PROGRESSION

Maria Ringvall

Solid tumors need to acquire new blood vessels to grow and become malignant. This is performed by angiogenesis, i.e. formation of new vessels from already existing ones. Many factors regulate tumor angiogenesis but the exact mechanisms are far from fully understood.

With the aim to get further understanding of the mechanisms that regulate physiological and pathological angiogenesis we use zebrafish and mouse as *in vivo* models. As a model for spontaneous tumor development we use the RIP1-Tag2 mouse where the tumors develop through a series of well characterized steps thought to mimic the way clinically observable tumors emerge. We evaluate genetically modified mice and cells in this model by comparing tumor vasculature and progression. Functional and/or spatiotemporal shifts in angiogenic status and tumor progression can then be further studied both *in vivo* and *in vitro* to unravel the mechanisms by which certain cells and molecules act.

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SERGLYCIN PROTEOGLYCAN AND MAST CELLS AS REGULATORS OF TUMOR ANGIOGENESIS

Nashwan Asmail, Sandra Andersson, Raja Sekhar Athoor, Grzegorz Furman, Andrew Hamilton, Ananya Roy and Maria Ringvall

Serglycin proteoglycan (SGPG) is an intracellular proteoglycan clad with heparin and/or chondroitin sulfate glycosaminoglycan chains. This proteoglycan is mainly expressed by cells that belong to the immune system. Although several cells that normally express SGPG are slightly affected by loss of expression, the most prominent phenotype in mice that lack SGPG is a mast cell defect with severely compromised storage of proteases and other mast cell mediators. The mast cell proteases act on extracellular matrix components and modulate the activity of other enzymes and high numbers of MCs in a tumor biopsy is generally correlated with active angiogenesis and a poor prognosis for the patient.

By investigation of tumor-bearing mice that lack SGPG or mast cells we assess the importance of these factors during tumor progression. To this end we examine different parameters such as angiogenic status, tumor volume and immunologic profile.

THE EFFECT OF HEPARAN SULFATE AND HEPARAN SULFATE MIMETICS ON ANGIOGENESIS

Andrew Hamilton, Benedikt von der Heyde, Nashwan Asmail and Maria Ringvall

The most abundant polysaccharide in the mammalian body is the sulfated glycosaminoglycan heparan sulfate (HS). Several signaling pathways involved in angiogenesis, such as FGF, VEGF and PDGF are regulated by HS that bind the ligand and/or act as a co-receptor. The degree, spacing and pattern of HS-sulfation regulate the capacity to bind different proteins.

We ar investigating how impaired endothelial HS-production affects tumor progression in the RIP1-Tag2 model. Of particular interest is how the angiogenic switch, i.e. the onset of vascular growth in dysplastic premalignancies, is affected.

We are also investigating the effect of small designed HS-mimetics on angiogenesis. To this end we use zebrafish as model organism for physiological angiogenesis and tumor-bearing mice for studies of pathological angiogenesis.

In another part of the project we investigate the functionality of the interaction between the endogenous angiogenesis regulator histidine-rich glycoprotein and HS for regulation of angiogenesis.

WHAT ARE GLYCOSAMINOGLYCANS GOOD FOR?

Dorothe Spillmann

Why does each cell in our body produce its own, sophisticated set of glycosaminoglycans (GAGs), a process that requires a battery of enzymes and a lot of energy? When GAGs are totally absent, organisms cannot develop beyond a few days of embryogenesis. When these carbohydrates are structurally altered due to changes in their biosynthetic production, serious consequences can be seen as *e.g.* failed organogenesis. In pathological situations of an organism altered structures may be encountered in parallel to disturbed homeostasis. Thus, one may wonder whether alterations are cause or result and whether and how GAGs can affect the organism in health and disease.

We see GAGs, predominantly heparan sulfate (HS) and chondroitin sulfates (CS), as tuners of molecular interactions at cell surfaces and in the matrix to allow for robust cellular interplay to create functional tissues. To approach our hypotheses we analyze structural features of GAGs from different sources, during various physiological and pathological conditions and correlate structural with functional properties apparent in these situations. We also deliberately modulate the expression of GAGs in model systems to check the influence of qualitative and quantitative structural changes on cellular functions. Such we can analyze how cells are affected by altered structures and how these changes translate at organism level during specific phenomena, *e.g.* regeneration processes in invertebrates.

Thus, our goals are to elucidate the underlying mechanisms how HS and CS structures modulate cellular behavior and communication, of critical importance to understand the control of physiological and pathological processes in multicellular organisms.

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HOW DO GLYCOSAMINOGLYCANS MODULATE CELLULAR FUNCTIONS?

Anna Eriksson, Dorothe Spillmann

One of our main foci is to understand how the propagation of an extracellular stimulus generated outside the cell, *e.g.* by a growth factor or the cellular contact to the surrounding, is affected by GAGs that can act as co-receptors for diverse ligands. How does the presence of GAGs affect the reception and propagation of stimuli from out- to inside? Are these features affected by how the GAGs are presented, where and how they are localized at the cell surface, in the matrix or released from their core protein such as seen by action of degrading enzymes *e.g.* released by cancer cells that pave their way to be able to metastasize? We have been able to show a direct role of structural features of HS chains when cells are stimulated by a growth factor, *e.g.* fibroblast growth factor (FGF) and

soluble chains to rescue HS-deficient cells. These effects may in turn be different when chains are attached to their core protein anchored in the plasma membrane or the matrix. We have therefore developed a cellular model system to characterize these effects: With different isoforms of the core proteoglycan syndecan expressed in various 'backgrounds' of GAG biosynthesis we now study the impact of the core protein, the role of different types of GAG chains and their structural features, on the propagation of extracellular stimuli (growth factor stimulation, adhesion) on the intracellular signals developed and the resulting cellular activities (*e.g.* proliferation, migration, contraction).

WHAT ARE THE FUNCTIONS OF GLYCOSAMINOGLYCANS IN LIMB REGENERATION

Rashmi Ramachandra, Ramesh Babu Namburi, Ulf Lindahl, Dorothe Spillmann

In contrast to a mammal, brittle stars have no problem to regenerate a limb. Brittle stars are stellate marine invertebrates found in most parts of the world. A majority of these animals have the capacity to autotomize their arms upon predator action followed by regeneration of the lost limb. This regeneration process resembles at least in part a recapitulation of developmental processes. What is the difference between them and us that they can regenerate lost arms while we don't? Are GAGs a part of the answer? We use brittle stars as models after having identified an extremely highly sulfated type of CS chains in one of the brittle star species [collaboration with M. Thorndyke, Kristineberg, GU]. Due to the exceptional structure of these GAGs, correlated with an exceptional limb regeneration capacity, we sought to study the structure/function relationship of these GAGs in the process of limb recovery. We therefore induced experimental autotomy in arms and followed the regeneration process. An increase in sulfation of GAGs was observed during progression of regeneration. Conversely, regeneration experiments with interference in biosynthetic sulfation resulted in a dramatic impairment of arm regeneration by severely affecting cell proliferation. Combined, these findings suggest that CS indeed have an important role in the molecular processes taking place during the regeneration of arm structures. We therefore aim at characterizing the molecular interactions of brittle star GAGs with factors and structures important during arm regeneration and to identify the corresponding biosynthetic genes to study their regulation during these processes.

WHEN MICROBES HITCHHIKE GLYCOSAMINOGLYCANS: FOR THE GOOD AND FOR THE BAD

Ramesh Babu Namburi, Dorothe Spillmann

As GAGs are prominently exposed on every cell in the body it is not surprising that microbes also make use of these structures to interact with their hosts. During the past years we have mainly focused on studying the effect of host-microbe interactions in order to develop antagonists for GAG based receptors and prevent or reverse microbial attack while avoiding overt interference with endogenous processes. We have earlier identified HS and CS motifs that are used by the malaria parasite *Plasmodium falciparum* [collaboration with A. Leitgeb, Dilaforette, and M. Wahlgren, KI, Stockholm], herpes virus [collaboration with T. Bergström and E. Trybala, Gothenburg University] and human

papilloma virus HPV [collaboration with H.C. Selinka, Johannes Gutenberg University, Mainz and M. Sapp, LSU Health Sciences Center, Shreveport, Louisiana, USA]. As a consequence of our earlier results adhesion competitors have been developed for malaria treatment and co-crystallization trials are ongoing for the parasite PfEMP1 protein. Also a co-crystal between the papilloma capsid protein L1 and heparin oligosaccharides has been successfully characterized. Lately, we have started collaboration with O. Berteau (INRA, Jouy-en-Josas, France) to characterize enzymes needed by symbiontic bacteria to make use of the host GAGs. Sulfatases are among such enzymes that commensal bacteria use for their survival. The main goal of characterizing these types of enzymes is to improve our understanding of successful host-microbe symbiosis, but also to gain valuable analytical tools.

CHARACTERIZATION OF GLYCOSAMINOGLYCANS

Dorothe Spillmann

The possibility to analyze GAG structures from different sources is a crucial requirement to correlate structure/function aspects of GAGs in different situations. We thus have a major interest to be able to characterize cells or tissues for their GAG production under different conditions. Therefore we continuously develop our high-throughput analysis technique for compositional analyses to further applications and optimize for diverse sample sources. As complementation of our analytic possibilities we are collaborating with J. Bergquist and M. Ramström Jonsson at the Dept. of Chemistry, UU, to also set up mass spectrometry based analytic tools for GAGs.

Earlier we have shown a remarkable tissue specificity of HS structures in adult mammals and looked at the evolutionary development of HS and CS in different tissues [collaboration with J. Ledin, EBC, UU]. We have also expanded these analyses looking into aging processes [collaboration with J.P. Li]. Every cell produces its own glycome, *i. e.* selection of glycan structures. Yet, if GAGs are meant to help in modulation of cellular activities one would assume that different requirements of the body would ask for adaptation of structures in order to fulfill the correct purpose and conversely that these structures may be changed in a 'wrong' way in pathological conditions and thus be reason for altered modulation resulting in disease. These questions we mainly follow by comparing GAG structures isolated from different specimen which have either been isolated from normal tissues, pathological biopsies or cell and tissue samples that have been exposed to altered growth conditions [diverse collaborations].

MEDICAL PROTEIN CHEMISTRY

Pia Ek, Åke Engström, Per Jemth, Birgitta Tomkinson

Proteins are essential to all life. They catalyse virtually all chemical reactions in the cell and they govern scaffolding and signalling. Protein chemistry is therefore central to all life sciences. In essence, results generated in fields such as genetics, cell biology and bacteriology can only be understood at a molecular level if we understand the structure and function of the proteins involved. Thus, for a profound understanding of any biological phenomenon a solid knowledge in protein science is imperative. Such basic knowledge is not only vital to gain through research but also crucial to convey to students in life sciences.

Here at IMBIM four groups pursue teaching as well as fundamental research on both enzymes and non-catalytic proteins. The Ek group studies a key mechanism in cellular function, namely regulation by phosphorylation. In particular, they examine histidine phosphorylation and the enzymes involved, a field where data is scarce but potential impact high. The Engström group provides a state-of-the-art proteomics facility as part of the technology platform for SciLifeLab in Uppsala. The power of their analyses lies in the extraordinary precision and accuracy of mass spectrometry, the highest in life sciences. The Jemth group looks at protein folding and protein ligand interactions and tries to unravel basic and general concepts about the action of proteins. In a second programme, the group focuses on proteins from human papillomavirus with the long term goal of preventing cancer caused by the virus. Finally, the Tomkinson group works on a huge and enigmatic enzyme, tripeptidyl-peptidase II, to reveal the molecular details of the catalysis as well as its physiological role. This enzyme is ubiqutious among eukaryotes and bigger than the ribosome!

The four groups ask different questions on various biological systems but share the common goal of understanding protein function at the level of molecular and atomic resolution. They also share the common goal of teaching undergraduate students fundamental biochemical principles and mechanisms. The aim is to make students in three different programmes (medicine, biomedicine and biomedical laboratory science) understand complex biological phenomena through basic concepts.

CHARACTERIZATION OF MAMMALIAN 14-kDa PHOSPHOHISTIDINE PHOSPHATASE

Pia Ek

Protein phosphorylation is a central mechanism of signal transduction in eukaryotic cells involved in all inter- and intracellular functions. The O-phosphorylation is performed by specific protein kinases, which transfer the -phosphate from ATP to an acceptor group of the selected amino acid residue in target proteins. Protein phosphatases make this process reversible.

The presence of phosphohistidine phosphate in eukaryotic cells has long been recognized but its physiological importance not yet understood. Phosphohistidine has been estimated to amount to as much as 6 % of the total phosphoamino acid pool in nuclei, indicating important functions. At least in slime mould, histidine phosphorylation is several times more abundant than tyrosine phosphorylation, although not as widespread as serine phosphorylation. However, histidine phosphorylation is much less studied than that of serine, threonine and tyrosine, and only a few phosphohistidine proteins have hitherto been identified. The major reason for this may have been methodological difficulties: phosphohistidine, which exists as one of two isomeric forms (3-phosphohistidine and 1-phosphohistidine) is labile at acid and neutral pH. Histidine phosphorylation would therefore easily escape detection, for instance with standard SDS-PAGE.

Published phosphohistidine kinases were not completely purified and there are some evidences that histidine is phosphorylated by other mechanisms, for instance by NDP kinase.

The mammalian 14-kDa phosphohistidine phosphatase, also denominated PHPT1, which we found more than 10 years ago by probing pig liver extracts with a phosphohistidine-containing peptide, has been further investigated. The localization of PHPT1-mRNA by Northern blot analysis revealed high expression in heart and skeletal muscle. An extended immunohistochemical analysis in mouse and human tissues using a PHPT1-specific polyclonal antibody was essentially consistent with the previously reported expression of corresponding mRNA of a few human tissues. In addition, several other tissues, including testis displayed a high protein expression. A salient result of the present investigation was the ubiquitous expression of the PHPT1-protein and its high expression in continuously dividing epithelial cells.

Phosphorylation of histone H4 has been described, and histone H4 histidine kinase has been isolated from different eukaryote cells; yeast, slime mould and from mammalian cells. Protein histidine phosphorylation has been described in cells that are highly proliferative and in regenerating cells. In pilot experiments in our laboratory we have phosphorylated recombinant histones H1 (one type), H2A, H2B, H3 and H4, using phosphoramidate, i.e. a chemical phosphorylation. We observed that all phosphohistones were dephosphorylated by PHPT1 and some of them more rapidly than the phosphorylated peptide succinyl-Ala-His(P)-Pro-Phe-*p*-nitroanilide used in our previous PHPT1-activity studies. Besides the recombinant histones, we have used histone H4 purified from commercially available sources. The corresponding phosphorylated H4 was dephosphorylated at a similar high rate.

We have developed an assay of PHPT1 using a histidine phosphorylated peptide from a newly described physiological target for PHPT1 – ion channel KCa3. This method can be used for assay of phosphohistidine phosphatase and kinase activity and makes it possible to search for proteins that are responsible for histidine phosphorylation.

Two mammalian splice variants of PHPT1 have been cloned and expressed in E coli and yeast and only the wild type had phosphohistidine phosphatase activity. No expressed variants had kinase activity.

An earlier finding by me from research on phosphorylation in and by prostasomes, resulted in a promising method for determination of prostate cancer malignancy.

Members of the group during 2012

Ulla Beckman-Sundh, graduate student
Pia Ek, Professor
Elvy Netzel, Biomedical scientist
Örjan Zetterqvist, Professor em

Publications 2010 to 2012

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Agencies that support the work

The Swedish Agricultural Research Council
The Swedish National Food Administration

EXPRESSION PROTEOMICS

Åke Engström and Eva Andersson

This laboratory gives the scientific community an opportunity to at a low cost make use of recent developments in techniques and instrumentations for the analysis and identification of proteins. The facility for Expression Proteomics is equipped with 2-D electrophoresis systems, systems for post or pre-gel labeling of proteins, visible and UV light scanners, software for image analysis, spot picking systems, semi automated spot processing and MALDI-TOF/TOF instrumentation. The facility has expertise for 2-D analysis, mass spectrometry, image analysis, data base searches and general protein chemistry.

Our area of work is analysis and comparison of proteomes, identification of proteins in protein spots/bands by mass spectrometry, analysis of expressed proteins for quality control and analysis of proteins for post-translational modifications. The facility is open for all scales of problem solving or analysis, although the capacity for 2D gels might be a limiting factor for very large undertakings.

The service is primarily intended for identification of proteins from species with large numbers of genes or proteins characterized. The facility has in addition a limited capacity for de novo sequencing of proteins from any species. The service includes straightforward methods for characterization of expressed recombinant proteins. Considering the low cost for analysis this is highly recommended to avoid the potential risk of doing experiments with the "wrong" or modified protein. An MS analysis of intact expressed protein and a peptide mapping with MS give much better confidence than a simple SDS-gel analysis. If suitable for our techniques and knowledge we provide analysis of any type of sample

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STRUCTURE-FUNCTION RELATIONSHIPS OF PROTEINS

Per Jemth

The ultimate goal of our research is to better understand fundamental structure-function and structure-reactivity relationships in proteins. Our research focuses on the nature and specificity of protein-ligand and protein-protein interactions, and also on protein folding, stability and allostery. We use protein engineering and biophysics to dissect the chemical reactions of proteins.

We use a number of model systems to address the fundamental questions we address. These model systems are small protein domains from modular proteins: PDZ domains, involved in scaffolding and signaling and the NCBD domain and pKID, which are intrinsically disordered and involved in transcriptional regulation. The lab is also running a project on proteins from human papillomavirus, namely E6 and E7. Certain strains of human papillomavirus cause cancer, for example cervical cancer, and this discovery was awarded the Nobel prize in physiology or medicine in 2008. The E6 and E7 proteins are so-called oncogenes, and the main culprits in the carcinogenesis. We want to understand the molecular details of their interactions with cellular proteins. Also check our web page: http://www.anst.uu.se/pje13912/.

Members of the group during 2012
Andreas Karlsson, PhD student
Celestine Chi, postdoc
Greta Hultqvist, PhD student
Jakob Dogan, postdoc
Maria Friberg, MSc student
Nakash Dinesh Shetty, MSc student
Per Jemth, Associate professor
Viveka Ramaiah, MSc student
Xin Mu, MSc student

International exchange during 2012:
Rait Kivi, Tartu

Publications 2010 to 2012
1. Di Matteo, A., Calosci, N., Gianni, S., Jemth, P., Brunori, M., Travaglini-Allocatelli, C. (2010) Structural and functional characterization of CcmG from Pseudomonas aeruginosa, a key component of the bacterial cytochrome c maturation apparatus. *Proteins* 78, 2213-2221.
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Agencies that support the work
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PROTEINS: FOLDING, STABILITY, INTERACTIONS AND ALLOSTERY

Andreas Karlsson, Celestine Chi, Greta Hultqvist, Jakob Dogan, Xin Mu

It is clear that many protein-ligand interactions involve structural rearrangements of the protein. These conformational changes are not restricted only to proteins with several subunits such as the classical example hemoglobin. Structural changes have also been detected in single domains. Sometimes the perturbation is very subtle and we want to investigate the role of such intradomain cross-talk. Other perturbations are not minor and the most extreme case of a conformational change would be folding of the protein, which we are also studying. Indeed, many proteins are even unfolded until they meet their binding partner.

One of the systems we are working on is PDZ domains. The PDZ domains are present in numerous proteins where they function as adaptors, governing binding to other proteins and thereby modulating, for example, signal transduction and scaffolding. One question we're addressing is how inter- and intramolecular conformational changes affect interactions between PDZ domains and their ligands. Another phenomenon we are interested in is proteins that fold only in the presence of their ligand. These so-called intrinsically disordered proteins are very common in the eukaryotic proteome, yet very little is known about them. We use different model systems: the ACTR and NCBD domains, pKID, and a disordered region of a PDZ domain.

HUMAN PAPILLOMAVIRUS AND CANCER: PROTEIN LIGAND INTERACTIONS

Andreas Karlsson, Celestine Chi, Maria Friberg, Nakash Dinesh Shetty and Viveka Ramaiah

The implication of human papillomavirus (HPV) in cancers of the uterine cervix has been firmly established biologically and experimentally and this discovery was awarded the Nobel prize in physiology or medicine in 2008. Other cancers are also caused by HPV. HPVs are classically divided into two groups: "low risk" and "high risk". The mucosal HPVs are considered "low risk" while the "high risk" is based on their prevalence ratio in cervical cancer, with HPV16 being the most common one. HPV utilizes mainly two proteins to immortalize infected cells, and they are called E6 and E7. These two proteins bind to a number of proteins involved in cell cycle regulation, for example p53 and retinoblastoma tumor suppressor. We are looking at the molecular mechanisms of interactions between HPV E6 and E7 proteins and their respective cellular targets. We hope that our basic research will open up new routes for treating HPV infection.

STRUCTURE, FUNCTION AND PHYSIOLOGICAL ROLE OF TRIPEPTIDYL-PEPTIDASE II

Birgitta Tomkinson

Intracellular protein degradation is as important for regulating the concentration of specific proteins in the cell as protein synthesis, but much less well characterized. Protein degradation is malfunctioning in a number of diseases such as cancer, muscle wasting and Alzheimers disease. Tripeptidyl-peptidase II (TPP II) is an important player in intracellular proteolysis, and our ultimate goal is to determine the specific physiological role of the enzyme in this process. TPP II is a huge enzyme complex with a widespread distribution in eukaryotic cells and the ability to cleave oligopeptides into tripeptides. Our main focus is a biochemical characterization of TPP II, in order to investigate how its substrate specificity is determined and how oligomerization is regulated. We are also investigating how expression of this enzyme varies in different cell types. These investigations will provide a basis for future drug discovery efforts. Since TPP II appears to be important for inactivation of the neuropeptide cholecystokinin and also for tumour progression, it is a potential drug target.

Members of the group during 2012:
Birgitta Tomkinson, professor
Jarmila Nahalkova, Ph. D. Researcher

Project workers during 2012
Sofia Gustafsson, "Expression and purification of murine tripeptidyl-peptidase II"
Therese Rydén, SOFOSKO-student, "Quantification of tripeptidyl-peptidase II in blood samples; investigation of a potential tumor marker"

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Agencies that support the work
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INVESTIGATION OF STRUCTURE AND FUNCTION OF TPP II

Birgitta Tomkinson, Sofie Gustafsson

This project focuses on the relationship between structure and function in TPP II. These studies are important not only for understanding the physiological role of the enzyme, but also in designing drugs targeting TPP II.

The major focus of the structure/function project has been on the endopeptidase activity of the enzyme. This activity is very slow compared to the exopeptidase activity (i.e. the release of tripeptides). The substrate specificity in particular has been studied, and these studies will continue in order to provide a basis for development of a specific substrate for the endopeptidases activity. Further studies include the pH-dependence of TPP II from three species with two different substrates. The results so far have given some insights into the structure of the active site, and have been expanded with experiments on point mutations.

CHARACTERIZATION OF TRIPEPTIDYL-PEPTIDASE II AND INVESTIGATION OF ITS POTENTIAL AS A TUMOUR MARKER

Jarmila Nahalkova, Therese Rydén, Birgitta Tomkinson

TPP II activity is increased in some cancer cells e.g. Burkitt´s lymphoma, and an overexpression of TPP II increases the risk for chromosomal damage as the enzyme appears to protect tumour cells from apoptosis. Therefore TPP II could be a potential marker for malignant tumours. In order to investigate this, a screening method for the expression of TPP II will be developed. A real-time PCR method has been developed in order to measure the amount of mRNA encoding TPP II in different tumour cells. In addition, an assay measuring enzyme activity has been evaluated and will be used for comparison of results. The different methods will then be compared to determine if the amount of mRNA, protein or active enzyme is correlated to tumour malignancy. The amount of TPP II will then be quantified in blood samples from healthy individuals and patients with different haematological diagnoses. This investigation is underway. Furthermore, activity-based probes are used as an additional tool for the identification of TPP II and other potential tumour markers in leukemia cells.

As a step towards understanding the physiological role of TPP II and its potential role in tumour progression, a project aimed at identifying potential interaction partners for TPP II in normal cells and tumour cells has recently been started.

TUMOR BIOLOGY

Anna-Karin Olsson, Pär Gerwins, Christian Sundberg, Staffan Johansson, Aristidis Moustakas, Kristofer Rubin

The collective efforts of the Tumor Biology unit concentrate on the dynamic interplay between malignant and surrounding cells within the tumor microenvironment. This focus follows the modern trend in cancer research that has steadily shifted from the classical studies of genetic alterations that drive the process of tumorigenesis to the study of the tumor tissue as an integrated system that supports tumor growth, local and distal expansion in the human body. Despite the importance of genetic mutations accumulating in cancer cells, the understanding of physiological processes and their deregulation in the tumor microenvironment raise much promise in the development of prognostic markers and novel targeted therapies.

The research projects of the unit aim at understanding how tumors recruit their blood vessels and how such processes attract various infiltrating cells that promote tumor progression (*Olsson, Sundberg*). The importance of integrin signaling and matrix remodeling is investigated in order to explain the physiological role of tissue tension in the expanding tumor (*Johansson, Gerwins*). Tumor progression is accompanied by a continuous plastic adaptation of the malignant and surrounding cells. Processes of cell de- or trans-differentiation, such as epithelial-mesenchymal transitions, and related fibrotic reactions that alter the physiological state of the tumor are also investigated (*Moustakas, Olsson, Rubin*). Translational efforts based on the above studies focus on novel strategies that can improve the uptake of anti-cancer drugs by growing tumors (*Rubin*). Finally, additional approaches towards novel cancer therapy focus on manipulation of the tumor vasculature by therapeutic vaccination and on inhibition of processes of trans-differentiation (*Moustakas, Olsson*).

MECHANISMS OF TISSUE VASCULARIZATION

Pär Gerwins

Neovascularization is a prerequisite for normal physiological processes and for development of human disease. The goal for the reseach group is to define mechanisms that regulate angiogenesis and tissue vascularization in order to understand disease development and to define new therapeutic targets. A combination of in vitro models and in vivo animal experiments are used which allows validation of basic findings in more complex models. We have developed a novel in vivo angiogenesis assay and discovered a potentially important and novel mechanism of tissue vascularization. Tensional forces generated by myofibroblast mediated contraction of wounds mediate and direct translocation of neovessels which are pulled from the pre-existing vasculature as loops with functional circulation within the expanding tissue. This new mechanism, that has been termed looping angiogenesis, is the basis for the future research in the group.

Group members 2012
Pär Gerwins, professor, group leader
Peder Fredlund Fuchs, PhD student
Ewa Kolosionek, post doc
Femke Heindryckx, post doc
Francois Binet, post doc

Publications 2010 to 2012

1. Kilarski WW, Petersson L, Fredlund-Fuchs P, Zielenski MS and Gerwins P. An in vivo neovascularization assay for screening regulators of angiogenesis and assessing their effects on pre-existing vessels. Angiogenesis, Aug 24. Dec;15(4):643-55 (2012).

FIBRIN DEGRADATION PRODUCTS AS REGULATORS OF NEOVASCULARIZATION AND FIBROSIS

Peder Fredlund Fuchs

Fibrinogen is a central protein in the haemostatic pathway that forms a provisional fibrin matrix in wounds, tumors and at sites of inflammation. Fibrin is continuously degraded by plasmin, which generates fibrin degradation products in the form of fragment E (FnE) and D-dimer. FnE has biological functions and stimulates proliferation and migration of endothelial and smooth muscle cells as well as induces angiogenesis by an unknown mechanism. Our hypothesis is that FnE released from the provisional fibrin matrix stimulates migration and differentiation of resident fibroblasts into myofibroblasts. In our model of looping angiogenesis the myofibroblast has a central role in generating biomechanical forces that in turn mediates neovascularization. The fibrin matrix would in this model be a natural source of stimuli for cell migration and neovascularization by forming a relatively stable gradient of FnE.

Preliminary results indicate that FnE is as potent as PDGF-BB in stimulating fibroblast migration. We also find that although FnE did not alter αSMA levels by itself it potentiated TGFβ induced myofibroblast differentiation as determined by a doubling of αSMA

expression compared to TGFβ alone. We have now initiated in vivo experiments using the chorioallantoic membrane assay and mice lacking fibrinogen to investigate if our in vitro findings can be translate to the in vivo situation. We are also purifying the putative FnE receptor.

These results have the potential not only to define fundamental mechanisms of angiogenesis in normal as well as pathological angiogenesis associated with diseases such as cancer, but also to provide novel therapeutic targets.

A LINK BETWEEN COAGULATION, TUMOR STROMA AND ANGIOGENESIS IN TUMOR GROWTH

Femke Heindryckx

An association between cancer and the haemostatic system has since long been recognized. There is a significant contribution of the coagulation system (fibrinogen, tissue factor, thrombin, factor X), the fibrinolytic system (plasminogen) and platelets to tumor growth and metastasis. Local deposition of fibrinogen/fibrin around tumor cells seems to be an early and universal event in most solid tumors. Fibrin(ogen) regulates proliferation, migration, apoptosis and expression of inflammatory mediators in tumor cells, fibroblasts, endothelial cells, and inflammatory cells through either integrin or non-integrin receptors. Furthermore, both fibrin and fibrin degradation products (FDPs) have been shown to support angiogenesis, consistent with the prevailing hypothesis that fibrin and its derivatives may promote tumor stroma formation by mechanisms that are comparable to those employed in normal tissue repair. Experiments in mice lacking fibrinogen have shown that the metastatic potential of tumor cells is greatly reduced in fibrinogen deficient animals. We are using fibrinogen knockout mice and inhibitors of coagulation and platelet function in xenograft and autochthonous (colon and liver cancer) tumor models to investigate the influence of fibrin(ogen) and and the coagulation system on tumor growth and metastasis. We have found that fibrin fragment E (FnE) stimulates migration and differentiation of myofibroblasts. Multiple monoclonal antibodies targeting FnE are under production with the goal to generate an antibody that binds and blocks the biological effects of FnE. Blocking antibodies will be administered to mice and its effects on angiogenesis, tumor growth, metastasis and stroma formation and wound healing analyzed. If successful, this could be the starting point for development of a novel treatment strategy.

BIOMECHANICAL FORCES AS REGULATORS OF NEOVASCULARIZATION AND LOOPINGANGIOGENESIS

Francois Binet

Biomechanical forces are important for embryo development as well as for reparative and pathological conditions in the adult. We have recently shown that tractional force generated during wound contraction directs and mediates angiogenesis and wound vascularization through a mechanism that has been termed looping angiogenesis. An important goal for our current research is to further explore the role of biomechanical regulation of neovascularization. To achieve this goal we use the mouse cornea as model system where sutures are placed in the cornea, which induces ingrowth of neovessels into the normally avascular cornea. By manipulating biomechanical forces surgically or by photochemical

cross-linking of the cornea we will be able to further study how biomechanical forces regulate tissue vascularization.

Time-lapse imaging has provided a great deal of information on vasculogenesis and angiogenesis during embryo development e.g. in Zebra fish embryos. However, since the mechanisms of angiogenesis in postnatal life likely are different from embryo development there is a need for time lapse studies in the adult during e.g. wound healing. The cornea model provides a suitable model system since the cornea is avascular and almost two-dimensional. We will use the suture model and follow the neovascular ingrowth using a contact endoscope, which will provide details of neovascularization and increase our understanding of looping angiogenesis.

NOVEL TGFβ REGULATED GENES IN MYOFIBROBLASTS

Ewa Kolosionek

TGFβ is considered to be one of the major inducers of αSMA expression and collagen 1 secretion in myofibroblasts and cancer associated fibroblasts through activation of the canonical Smad pathway that regulates transcription of a large number of genes. The objective is to analyze TGFβ regulated genes in primary human fibroblasts in a non-biased manner using cDNA micro array. As expected we find that TGFβ increase expression of e.g. αSMA, NOX4 and N-cadherin. However, we also find highly induced expression of genes not previously known to be TGFβ regulated. One of these new genes is up-regulated more than 50 times after addition of TGFβ. Preliminary results suggest that knock-down using RNAi impair cell migration. Little is known on the function of the protein but high expression has been observed in malignant glioma.

ADHESION-DEPENDENT CELL SIGNALING

Staffan Johansson

Adhesion of cells to specific proteins in the extracellular matrix contributes to the organization of tissues. In addition, it provides the cells with information about the surrounding environment, which is important for their migration, differentiation, and proliferation. As the main adhesion and migration receptors of cells, integrins are potential targets for regulation of several adhesion-related events of clinical relevance, such as wound healing, angiogenesis, thrombus formation, leukocyte extravasation to inflammatory sites, and tumor metastasis. Our work is focused on integrin signals involved in adhesion-dependent cell survival and proliferation.

Members of the group during 2012
Staffan Johansson, professor
Rajesh Gupta, postdoc. (until 120730)
Anjum Riaz, PhD student
Birgitta Wärmegård, technician
Kathrin Zeller, PhD student/postdoc

Publications 2010 to 2012

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Agencies that support the work
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REGULATION OF SURVIVAL, MIGRARATION, AND CYTOKINESIS BY INTEGRINS

Rajesh Gupta, Anjum Riaz, Birgitta Wärmegård, Kathrin Zeller

a) During cell adhesion to extracellular ligands, integrins generate intracellular signals. A key step in integrin-mediated signaling is the activation of PI3 kinase, a reaction required for the anti-apoptotic effect of adhesion as well as for several other signaling pathways. We have identified p110α as the catalytic isoform of the PI3 kinase family that is activated by β1 integrins. Further, we showed that the PI3 kinase-dependent Ser473 phosphorylation of AKT1/2 significantly contributes to adhesion-induced survival signals. Analysis of the requirement of RICTOR, ILK, and PAK1/2 for AKT Ser473 phosphorylation downstream of β1 integrins, LPA receptors (G protein-coupled), PDGF and EGF receptors (tyrosine-

kinases), and heparanase receptor revealed that the mechanism is more complex than commonly depicted and varies depending on the stimulated receptor.

Inhibition of p110α PI3K activity was found to suppress the rate of actin polymerization induced by b1 integrin stimulation. By monitoring lamellipodia protrusion during cell attachment with TIRF microscopy, we have developed an assay to analyze the mechanism by which integrins trigger the polymerization reaction.

A main function of integrins is to serve as "mechanoreceptors". We study the role of integrins for signaling responses to external force acting on the cell as well as to actomyosin-based intracellular force, with a focus on the generation of reactive oxygen species. Our results show that cellular responces generally refered to as "integrin signals" are actually composed of separate sets of reactions triggered by different types of integrin stimulation, i.e. by ligand-binding during cell adhesion and mechanical force during cell stretching.

b) Cytokinesis of normal adherent cells requires signals from integrins, and the lack of such signals in detached cells cause formation of binucleated cells. Our data shows that a new round of the cell cycle still will proceed in the absence of cytokinesis, and that cytokinesis will resume uncoupled from karyokinesis if such cells reattach. Although most of the reattached cells appear to divide successfully, we found that the uncoordinated karyokinesis-cytokinesis results in increased numbers of permanently binucleated cells, known to cause aneuploidy and chromosomal instability. The significance of transient cell detachment and chromosomal instability will be further studied.

c) Spheroid cultures of transformed cells have been found useful as in vitro models for several aspects of avascular tumor growth. Compact spheroid morphology has been strongly correlated with invasive growth *in vivo*. Our data suggest that the compactness and size of spheroids is differently regulated in β1-expressing and β1-/- spheroids. β1 integrins strongly enhance the compactness of spheroids via regulation of myosin light chain phosphatase (MYPT1) and cofilin phosphorylation levels, and β1 integrins may therefore promote tumor invasiveness. On the other hand, the absence of β1 integrins resulted in excessive growth of "loose" spheroids from which cells often detached. Such properties are also likely to cause tumor spreading, e.g. by dissemination of micro-metastases into body cavities. In order to use β1 integrins as targets for cancer treatments, the observation that these receptors actually may have both positive and negative effects on tumorigenesis has to be taken into account.

d) See Catharina Svensson for a collaborative project on a mouse model for human adenovirus in tumor therapy.

SIGNAL TRANSDUCTION AND EPITHELIAL PLASTICITY

Aristidis Moustakas

Our research program covers several aspects of signal transduction and basic cancer biology. Special attention is given to the developmental process of epithelial-mesenchymal transition (EMT) and its links to tumor metastasis and cancer stem cell biology. EMT confers upon cancer cells capacities that are required for metastasis. We want to explain how the EMT process contributes to the maintenance of cells that carry tumor-initiating and metastasis-initiating capacities. Our efforts to develop novel chemicals that inhibit EMT promise to take our research to more applied areas of medical science.

From the signal transduction perspective, the lab focuses on the Transforming Growth Factor β (TGFβ) pathway and on nuclear events such as regulation of transcription. In addition, we study cytoplasmic signaling events and in particular mechanisms of cell polarity regulation by the tumor suppressor kinase LKB1 and its downstream effectors, the AMP-regulated kinase (AMPK) family.

TGFβ regulates cellular processes such as cell growth, differentiation, and tumorigenesis via a group of proteins known as Smads and via several intracellular kinase and GTPase pathways. The Smads transmit signals from type I and type II TGFβ receptors on the cell surface and into the cell nucleus, where they regulate gene expression. While previous work by the team demonstrated how Smads shuttle in and out of the nucleus in response to changes in TGFβ signaling, we now focus more on the activity of Smads inside the nucleus. TGFβ signaling has a complex impact on tumorigenesis. The pathway suppresses the growth of early-stage tumors by inhibiting cell growth or by prompting cells to undergo apoptosis, but nevertheless drives tumorigenesis in late-stage tumors. We investigate the function and regulation of various TGFβ-responsive genes by combining functional experiments with global gene expression analysis in several cell models of epithelial cells. This approach has allowed us to decipher key steps in the genetic program that mediates tumor suppression or tumor progression in response to TGFβ.

Members of the group during 2012
Laia Caja, post-doc*
Jonathon Carthy, post-doc* (from Mar 2012)
Markus Dahl, PhD student*
Mahsa Shahidi Dadras, PhD student (from Dec 2012)
Kaoru Kahata, post-doc*
Varun Maturi, PhD student* (from May 2012)
Anita Morén, technician*
Aristidis Moustakas, lecturer*
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Masoud Razmara, post-doc* (till Dec 2012)
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Michael Vanlandewijck, PhD student* (till Feb 2012)
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Project workers during 2012
Mahsa Shahidi Dadras, project worker (from Mar till Sep 2012)
Ashish Kumar Singh, MSc student* (from Jan till Sep 2012)
Kalliopi Tzavlaki, project worker (from Aug 2012)

Angelos Heldin, project worker* (from Jun to Aug 2012)

* co-affiliated with the Ludwig Institute for Cancer Research (LICR).

International exchange 2012
Yukari Okita, visiting PhD student, Univ. of Tsukuba, Japan.

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Agencies that support the work

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ENZYMATIC REGULATION OF TGF-β/SMAD SIGNALING IN THE NUCLEUS

Markus Dahl, Varun Maturi, Anita Morén and Yukihide Watanabe

Continuing our activity in previous years, we attempt to identify novel enzymatic regulators of Smad function in the nucleus that operate at the chromatin level. We also analyze Smad protein turnover and the role of ubiquitination in this process, by focusing on HECT-domain ubiquitin ligases and the prolyl-isomerase Pin1. Smads can modify the expression of a few hundred genes by cooperating with different DNA-bound factors, some of which act by modifying the structure of chromatin. We focus on enzymes such as the tumor suppressor kinase LKB1 and the ribosyl-transferases PARP-1 and PARP-2, which regulate nucleosome assembly and transcriptional initiation and elongation. We want to understand how such nuclear enzymes organize integrated biological responses by modulating the activity of TGF-β signaling. We also attempt to understand how post-translational modifications of Smads may change between normal and cancer cells and try to explain abnormal behavior of the pathway in cancer. A major technique that we apply widely in these studies is the in situ proximity ligation, which provides us with greater

sensitivity of detecting the endogenous proteins interactions and post-translational modifications.

This work has been partially carried in collaboration with Dr. Carl-Henrik Heldin of the Ludwig Institute for Cancer Research (LICR) at Uppsala University, Dr. Ulf Landegren of the Department of Genetics and Pathology of Uppsala University, Dr. Michael Hottiger of the Univ. of Zurich, Switzerland and Dr. Takeshi Imamura, Ehime University, Japan.

REGULATION OF TGF-β/BMP RECEPTOR SIGNALING BY PROTEIN KINASES

Jonathon Carthy, Mahsa Shahidi Dadras, Anita Morén, Erna Raja, Masoud Razmara and Michael Vanlandewijck

Via genome-wide transcriptomic analysis, we previously found that the *salt-inducible kinase* (*SIK*) and the *Nuak2* genes represent new TGF-β-responsive genes, and are AMP-regulated kinase (AMPK) members, best known as substrates of the master kinase and tumor suppressor LKB1. SIK regulates turnover of the TGF-β receptor after ligand binding by cooperating with Smad7 and the Smurf ubiquitin ligases. Peutz-Jeghers Syndrome (PJS) patients develop benign hamartomatous polyps in early age and are predisposed to intestinal or other forms of cancer in adult life. PJS is caused by loss-of-function mutations in the *LKB1* gene. PJS hamartomas resemble those in Juvenile Polyposis Syndrome (JPS) patients, who inherit inactivating mutations in the *Smad4* or the BMP type I receptor, *BMPR1A* genes. We try to uncover the molecular links between TGF-β, BMP and LKB1/AMPK signaling by focusing on mechanisms of receptor function and trafficking. We have proven that LKB1 negatively regulates the BMP type I receptor ALK2, a process important during *Drosophila* organogenesis and lung cancer progression.

This work has been partially carried in collaboration with Dr. Carl-Henrik Heldin of the LICR at Uppsala University, Dr. Patrick Micke, Department of Immunology, Genetics and Pathology at Uppsala University, Dr. George Pyrowolakis of Freiburg University, Germany and Dr. Ola Söderberg of the Department of Genetics and Pathology of Uppsala University.

MOLECULAR MECHANISMS OF EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

Jonathon Carthy, Mahsa Shahidi Dadras, Kaoru Kahata, Varun Maturi, E-Jean Tan and Michael Vanlandewijck

In an effort to explore the relationship between EMT and cancer progression we found that long-term exposure of mammary epithelial cells to TGF-β promotes EMT and enhances tumor growth due to enhanced secretion of chemokines. Tumors obtained from cells that had undergone EMT were enriched in cells that could differentiate into mammary epithelial and thus might resemble mammary stem cells. Deeper molecular analysis of the EMT process established that the nuclear factor HMGA2, whose levels are induced by TGF-β, can bind directly to Smad proteins and together Smads and HMGA2 induce expression of major factors that promote EMT, such as Snail and Twist. This work has also revealed that HMGA2 and Smads may cause global chromatin remodeling and DNA methylation patterning during EMT, a direction that we currently follow using genome-

wide transcription factor location analysis. In addition, we analyze the role of LKB1 and specific members of the AMPKs (SIK) in regulating critical aspects of the EMT process, including cell polarity. Finally, we perform screens for novel inhibitors of EMT.

This work has been partially carried in collaboration with Dr. Carl-Henrik Heldin of the LICR at Uppsala University, Drs. Andrew Shiau and Timothy Gahman of the LICR at San Diego, USA, Dr. Martin Stöter of the Max Planck Institute, Dresden, Germany and with Drs. Christer Busch, Patrick Micke and Fredrik Pontén of the Department of Immunology, Genetics and Pathology of Uppsala University.

LINKS BETWEEN INVASION AND SELF-RENEWAL OF TUMOR INITIATING CELLS

Laia Caja, Kaoru Kahata, Varun Maturi, E-Jean Tan and Kalliopi Tzavlaki

HMGA2 has recently been shown to regulate the ability of normal brain stem cells to self renew. We therefore try to analyze the role of TGF-β and HMGA2 in the survival and tumor-initiating potential of tumor-initiating cells of the breast and brain (glioblastoma multiforme, GBM). We study the interplay between the nuclear chromatin factors HMGA2 and CTCF and the regulation of micro-RNA genes, processes that seem to be critical for the establishment of self-renewal of tumor-initiating cells. In addition, we performed a genome-wide screen for mRNAs expressed under the control of the differentiation factor BMP-7, which suppresses GBM tumorigenesis. We analyze the function of selected genes that mediate the tumor suppressor action of BMPs in the GBMs. Surprisingly we found that BMP signaling induces expression of the transcription factor Snail, which then causes astrocytic differentiation of the GBMs, enhanced invasiveness and loss of self renewal capacity by the GBM cancer stem cells. We also developed mouse xenograft models based on human GBMs that have been engineered to express the EMT regulator, Snail. In this project we aim at understanding links between tumor cell invasiveness and tumor-initiating capacity in vivo.

This work has been partially carried in collaboration with Dr. Carl-Henrik Heldin of the LICR at Uppsala University, Drs. Lene Uhrbom, Bengt Westermark and Karin Forsberg-Nilsson of the Department of Immunology, Genetics and Pathology of Uppsala University.

TUMOR VASCULAR BIOLOGY

Anna-Karin Olsson

Deregulated angiogenesis (formation of new capillary blood vessels) has been implicated in a number of pathological processes, for instance rheumatoid arthritis, retinopathy and tumor growth, and contribute to progression of the disease. To prevent or reduce angiogenesis in these situations is therefore of clinical interest. A small number of drugs that prevent signaling via vascular endothelial growth factor (VEGF), an important growth factor for blood vessels, have been approved for clinical use, both for the treatment of macula degeneration and different types of cancer. However, the benefit for the treated cancer patients is still relatively limited and therapy-induced resistance commonly develops against these drugs. The aim of our group is to develop new and effective treatment strategies for cancer by targeting its vasculature. One of our projects is focused on the development of therapeutic cancer vaccines directed specifically at molecules expressed by tumor vessels. We are also interested in the genetic and molecular mechanisms responsible for deregulated blood vessel formation. A special emphasis is put on the role of platelets, which have the capacity to promote angiogenesis and tumor growth. Cancer patients commonly display elevated platelet activation and have an increased risk of thromboembolic complications. This highlights the potential benefit of keeping platelet activity as low as possible in cancer patients, without increasing the risk of bleedings.

Members of the group during 2012
Jessica Cedervall, post-doc
Julia Femel, graduate student
Else Huijbers, post-doc (graduated May 11)
Anna-Karin Olsson, Assoc Prof, group leader
Yanyu Zhang, graduate student

Publications 2010 to 2012

Original articles

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The Medical Faculty, Uppsala University

TARGETING TUMOR VESSELS BY THERAPEUTIC VACCINATION

Julia Femel, Else Huijbers

A small number of molecules have been identified as specifically expressed by tumor blood vessels, either by the endothelial cells themselves or by the adjacent stroma. These molecules are interesting targets for therapeutic vaccination against cancer, since they are absent in normal tissue and not expressed by the actual tumor cells, which often escapes the immune system. We have developed a therapeutic vaccine against the extra domain-B (ED-B) of fibronectin. This 91 amino acid-domain, inserted by alternative splicing, is expressed during vasculogenesis in the embryo, but essentially undetectable under normal conditions in the adult. However, ED-B is highly expressed around angiogenic vasculature such as in tumorigenesis. Mice immunized against ED-B displayed a 70% reduction in tumor growth and a significantly reduced function of the tumor vasculature. These data show that tumor vascular antigens are highly interesting candidates for development of therapeutic vaccines targeting solid tumors. We are now continuing this work by immunizing against additional tumor vascular targets, alone or in combinations, to see if the efficacy of the vaccine can be enhanced. Moreover, we are addressing the effect of the vaccines on established tumors, which is the clinically relevant situation. Finally we are also investigating whether formation of metastases can be prevented/reduced by targeting tumor vessels by therapeutic vaccination.

HISTIDINE-RICH GLYCOPROTEIN IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

Jessica Cedervall, Yanyu Zhang

Histidine-rich glycoprotein (HRG; alternatively, HRGP/HPRG) has been identified as an angiogenesis inhibitor *in vitro* and *in vivo*. HRG has the capacity to reduce tumor growth and vascularization in mice. We are presently addressing the role of HRG in physiological and pathological angiogenesis using HRG-deficient mice, which are cross-bread to transgenic tumor models. A model that we use for this purpose is the RIP1-Tag2 mouse that develops orthotopic insulinoma through a multistep process. One of these steps is characterized by induction of angiogenesis, the so called "angiogenic switch", which renders the RIP1-Tag2 mouse an excellent model for studies of tumor angiogenesis. We have demonstrated that mice lacking HRG have an elevated angiogenic switch and display increased tumor growth, a finding that firmly establishes HRG as an endogenous regulator of pathological angiogenesis. Another transgenic model in our lab is the MMTV-PyMT model for breast cancer. These mice closely resemble the human disease and accordingly develop metastases. Using these models we are currently investigating the role of HRG in physiological and pathological angiogenesis, as well as the impact of HRG on tumor growth and metastasis.

THE ROLE OF PLATELETS IN TUMOR VASCULARIZATION

Jessica Cedervall, Yanyu Zhang

Platelets are anuclear cellular fragments, which play a crucial role in regulating blood hemostasis as well as non-hemostatic processes such as immunity, tumor metastasis and angiogenesis. In different experimental settings, platelets have been shown to stimulate angiogenesis. Our research is focused on the mechanisms by which platelets affect tumor vascularization, with a special focus on the role of HRG in this process. Mice lacking HRG display enhanced coagulation and increased platelet activation. By depleting HRG-deficient mice of platelets we could suppress the elevated angiogenic switch to the level found in wild-type mice. These data show that platelets are crucial mediators of the accelerated angiogenic switch in HRG-deficient mice. We are currently investigating the mechanism behind the enhanced platelet activation in HRG-deficient mice, as well as their role in pathological angiogenesis.

LOOSE CONNECTIVE TISSUES – POTENTIAL TARGETS FOR THERAPIES IN CANCER AND INFECTIOUS DISEASES

PI Kristofer Rubin

Loose connective tissue elements are present in all organs outside the central nervous system. They embed blood vessels and underlie mucosal surfaces and also constitute the stroma of carcinoma. During inflammatory processes leukocyte leave the blood vessels and enter the surrounding loose connective tissues. The composition of the interstitial matrix, *i.e.* the amount (concentration) and type of the fibrous scaffolding and ground substance, in concert with connective tissue cells determine the physical properties for convective and diffusive movement of molecules in the tissue. The loose connective tissue surrounding blood vessels was commonly thought of as a "passive" framework in the sense that its physical properties such as diffusivity, hydraulic conductivity, compliance and interstitial fluid pressure (IFP) remain fairly constant. This concept of a static and passive loose connective tissue has been challenged by recent research suggesting that IFP is "actively" controlled and thereby also fluid content and possibly fluid fluxes through tissues.

Together with prof. Rolf Reed at Bergen University in Norway we have proposed a mechanism for control of IFP *in vivo*. Our proposed mechanistic model holds that connective tissue cells apply tensile forces on ECM-fibers that in turn restrain the under-hydrated ground substance from taking up fluid and swell. A decrease in cellular tension on the ECM fibers allows the ground substance to swell and form edema. During this process negative IFP values can be recorded if refilling of the tissue with fluid is inhibited. Dermal IFP lowered after anaphylaxis can be normalized by instilments of platelet-derived growth factor (PDGF) BB or insulin. Our data suggest that whereas β_1-integrins participate in of maintenance fluid homeostasis, β_3-integrins participate in PDGF BB-induced IFP-recovery after inflammation-induced lowering of dermal IFP.

One obstacle in the pharmaceutical treatment of carcinomas is the poor uptake of anti-cancer drugs into the tumor tissue. We have shown that the IFP in carcinoma reflects conditions that form a barrier for penetration of low-molecular compounds such as chemotherapy into the carcinoma tissue. Lowering of carcinoma IFP by local treatment with prostaglandin E_1 or by systemic treatment with inhibitors of the PDGF or TGF-β systems lower carcinoma IFP. This lowering of IFP is paralleled by increases of the efficacy of conventional chemotherapy. We have spent considerable efforts in elucidating the mechanisms by which carcinoma IFP is controlled. Microarrays for gene expressions and other techniques such as immunohistochemistry, real-time PCR, imaging and cell analyses, all combined with physiological measurements in carcinoma grown in wild-type or transgenic animals have been adopted. We have a found a correlation with inflammatory processes and the architecture of the collagen network in the stroma.

Group members during 2012
Kais Algilany, PhD, Scientist
Per Bush, assistent
Maria Göthe, MSc, Research assistant
Renata Gustafsson, PhD, postdoctoral fellow
Per Olof Olsson, MSc, student

Vahid Reyhani, MSc, PhD-student
Lars Rask, PhD, professor in Medical Biochemistry
Kristofer Rubin, PhD, professor in Connective Tissue Biochemistry
Cecilia Rydén, MD, PhD, assoc professor, and senior consultant in infectious diseases (Until July)

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Agencies supporting the work
The Swedish Cancer Society, the Swedish Science Council and the Swedish Medical Association.

FIBROBLAST-MEDIATED COLLAGEN GEL CONTRACTION

Vahid Reyhani

Fibroblast-mediated collagen gel contraction is stimulated by PDGF-BB and inhibited by pro-inflammatory agents such as prostaglandin E_1 and interleukin-1. Recently, others and we have identified at least two mechanisms for cell-mediated collagen gel contraction. The collagen-binding integrins, $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ all mediate rapid contraction that proceeds in serum-free media. Contraction mediated by these integrins requires integrin-elicited

signaling. When the collagen-binding β_1 integrins either are absent or their signaling and/or activity perturbed, a second mechanism can become operative. This mechanism depends on the RGD-dependent integrin $\alpha_V\beta_3$ and is stimulated by *e.g.* PDGF-BB. Our data suggest that gel contraction can serve as an *in vitro* model for control of IFP *in vivo*. In general, our approach is to study the mechanisms by which tissue tension is controlled using the collagen gel contraction as an *in vitro* model system and verify the results by studies of fluid volume control in experimental animals. We focus on the importance of specific signal pathways and extracellular matrix proteins.

FLUID DYNAMICS IN CARCINOMA

Kais Algilany, Tomas Friman, Maria Göthe and Renata Gustafsson

Our data show that the collagen network architecture determines patho-physiological properties of a carcinoma. We have reported on a correlation between thinner collagen fibrils and low IFP in experimental carcinoma. Treatments with inhibitors of TGF-β or PDGF that reduce IFP and normalize fluid content also reduce collagen fibril diameters. We have furthermore reported that the small leucine-rich proteoglycan fibromodulin can play an important role in regulating the structure and function of the collagen network in experimental carcinoma. Recently, we have shown that carcinomas grown in integrin β_3 deficient animals have coarser collagen fibrils and fibers, higher IFP, increased fibromodulin expression and lower levels of extracellular water.

An important issue for our further research is to elucidate the molecular mechanism by which the collagen network architecture is controlled by collagen producing cells in a carcinoma. We are currently investigating how matricellular proteins controls assembly of collagen networks. In this context we also study the potential role of integrin $\alpha_V\beta_3$-binding matricellular proteins for collagen assembly.

The structure/function relationship of the (ECM) in carcinoma is an underdeveloped area that has an obvious clinical relevance. We collaborate with clinicians to validate our findings in clinical cases of carcinoma.

MECHANISMS OF OPTIMAL TISSUE REGENERATION VERSUS FIBROSIS AND THE ROLE OF THE MICROVASCULATURE

PI: Christian Sundberg

The main focus of this group is to understand the biology of blood vessels and their role in tumor formation and fibrosis. Fibrosis is a common denominator in a wide variety of diseases characterized by chronic inflammation including stroma formation in solid tumors, rheumatoid arthritis and inflammatory bowel disease, connective tissue diseases, atherosclerosis, heart failure, transplant rejection and wound healing to name a few. The progression of fibrosis in these diseases leads to the derangement of tissue architecture and subsequent failure of the organ. In many of these diseases current therapeutic approaches have only marginally contributed to cure and must be seen as approaches that delay the progression of the disease. However, in certain circumstances in the adult, diseased organs (for instance the kidney in glomeruloid nephritis, the liver after hepatitis, and the heart during ventricular hypertrophia) are capable of healing themselves with minimal damage to the tissue and its function. Tissue regeneration following damage to an organ during embryogenesis and infancy is also an example of tissue repair with minimal functional sequel. Thus, the body has mechanisms by which to adequately repair damaged organs. Why the body does not always achieve this, and what causes progression in one instance, and healing in another, is largely unknown and is one of the main subjects of study in the laboratory.

Group members
PI: Christian Sundberg, MD, PhD, associate professor, Senior Scientist at the Swedish Scientific Research Council, Medical Branch.

Post Doc. Position open
Project worker. Position open

Publications 2010 to 2012

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3. Rodriguez A, Friman T, Gustavsson R, Kowanetz M, van Wieringen T and Sundberg C. Phenotypical differences in connective tissue cells emerging from microvascular pericytes in response to over-expression of PDGF-B and TGF β1 in normal skin in vivo. Am J. Pathol. In Print.

1. DEFINING THE PERICYTE-FIBROBLAST LINEAGE AND THEIR COMMON STEM CELLS

We have published the novel concept that microvascular pericytes have the ability to differentiate into collagen type I producing fibroblasts, thereby coupling the process of

angiogenesis and fibrosis in a previously unrecognized way. Our research involves the isolation and study of stem cells that define the pericyte-fibroblast lineage, and to identify different stages of this differentiation process. Preliminary results show that five different stages are involved in this process. We will study differences in gene expression and gene products during this differentiation process using cDNA microarray techniques as well as proteomics (2-D gel electrophoresis). By defining this new lineage novel insights into the process of fibrosis and potential modulation will be identified.

2. INHIBITING THE PERICYTE-FIBROBLAST DIFFERENTIATION PROCESS

As a follow up project to point 1 we will devise an assay to study the differentiation of pericytes to collagen type 1 producing fibroblasts in a high throughput assay system. We will use a chemical library to attempt to identify compounds that modify this differentiation process. Furthermore, conditioned medium from certain tumor cell lines have an inhibitory effect on the differentiation process. We would like to identfy what this component in conditioned medium is.

3. GENE THERAPEUTIC APPROACH FOR STUDYING FIBROSIS AND BLOOD VESSEL FORMATION IN THE BODY

We have previously published a novel animal model by introducing VPF/VEGF into normal tissues by adenoviral vectors. These studies have led to the discovery of three previously unknown modes of angiogenesis. We will further study cell progression and events that occur during blood vessel formation and fibrosis in the body. To this effect gene therapy techniques using adenoviral vectors will be used in order to induce genes for growth factors that are believed to modulate the development of the tumor stroma. Growth factors will be introduced into normal and diseased tissues, both individually and in combination. Effects of these growth factors will be studied using advanced morphological and physiological techniques which are being developed. This approach might be used for treatment of heart disease as well as diseases in other arteries in the body resulting from arteriosclerosis and diabetes.

IMMUNOLOGY

Birgitta Heyman, Frida Henningson Johnson, Jenny Halllgren Martinsson, Kjell-Olov Grönvik

The cellular and humoral components of the immune system are crucial in our defense against foreign microorganisms. The central theme in our work is to try to understand how antibodies, complement, mast cells, dendritic cells and T cells are operating in concert to achieve an optimal immune response. We also try to understand what goes wrong when allergies develop. We are primarily working in mouse models using different transgenic, knock-out and knock-in strains but in addition in vitro techniques and human studies are used.

ANTIBODY FEEDBACK REGULATION

Birgitta Heyman

Antibodies in complex with their specific antigen can feedback-regulate antibody responses against this antigen. Depending on antibody class, affinity and type of antigen, complete suppression or 10-1000-fold enhancement of the in vivo immune response can be seen. Both passively administered and actively produced antibodies are effective, suggesting a biological role. One of the most successful clinical applications of modern immunology is Rhesus prophylaxis, where administration of suppressive IgG anti-RhD prevents Rh-immunization in Rh negative mothers carrying Rh postive fetuses. This antibody treatment has almost eradicated hemolytic disease of the newborn in industrialized countries. In spite of this successful treatment, the mechanisms behind feedback regulation are poorly understood. An immune complex is composed of antigen/antibody/complement (if the antibody is able to activate complement). Such immune complexes can bind to the B-cell receptor, Fc-receptors (FcR) and complement receptors (CR). Ligation and co-ligation of these receptors on the B cell surface can negatively or positively regulate the B cell. Increased uptake of complexed antigen by antigen-presenting cells via FcRs or CRs can enhance T helper cell activation. Follicular dendritic cells (FDC) do not express MHC-II molecules and do not present antigen to T cells. They are interspersed in the B cell follicles of the spleen and lymph nodes and interact closely with B cells. Since FDC express both FcR and CR, they may capture immune complexes and act as a concentration device, facilitating antigen recognition by the B cells. A novel interesting function of antibodies is to transport antigen into the optimal locations in secondary lymphoid organs. The main objective of our research is to clarify the mechanisms behind antibody feedback regulation, both during a normal immune response and in autoimmune diseases and allergies.

Members in the group during 2012
Anna Bergman, PhD student
Joakim Bergström, project assistant
Zhoujie Ding, PhD student
Birgitta Heyman, professor, group leader
Christian Rutemark, post-doc
Lu Zhang, PhD student
Annika Westin, technician

Publications 2010 to 2012

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Agencies that support the work
The Swedish Research Council, von Kantzow's Foundation, Eriksson's Foundation, Hesselman's Foundation, King Gustaf V:s 80 Year Foundation, Agnes och Mac Rudbergs stiftelse, Uppsala University.

MECHANISMS FOR COMPLEMENT-MEDIATED REGULATION OF IMMUNE RESPONSES

Christian Rutemark, Anna Bergman, Zhoujie Ding, Lu Zhang, Birgitta Heyman

Complement receptors 1 and 2, CR1/2 are important for the production of antibodies since CR1/2-knock-out mice have very poor antibody responses. Activation of complement via the classical pathway explains the influence of complement on antibody responses. Antibodies, and in particular IgM, are the activators of the classical pathway. We have shown that although C1q-knockout mice have abrogated antibody responses, knock-in mice which produce IgM with a point-mutation making the IgM-molecule unable to activate complement, have near-normal antibody responses. This is a surprising finding, demonstrating that something else than antibodies must activate the classical pathway. We are now trying to identify which substance does activate C1q in these situations and where in the body the antigen becomes covered with activated complement factors.

The mechanism by which complement is required for antibody responses is not known. CR1/2 expressed on B cells play a central role in responses to antigens administered alone. In addition we study the ability of IgM and IgG3 antibodies to upregulate antibody responses. It is known that this requires complement activation and several mechanisms are possible, such as transport of antibody-antigen complexes on marginal zone B cells from the marginal sinus into B cell follicles and co-crosslinking of membrane-bound IgM and CR1/2 on the surface of B cells. Using bone marrow chimeras and confocal microscopy we hope to clarify which cells are involved in IgM- and IgG3-mediated enhancement and how these antibodies affect localization of the antigen.

MECHANISMS FOR IgG-MEDIATED SUPPRESSION OF IMMUNE RESPONSES

Joakim Bergström, Anna Bergman, Birgitta Heyman

Another project aims at understanding the mechanisms behind IgG-mediated suppression of antibody responses. Purified IgG, prepared from serum of mice hyperimmunized with

sheep erythrocytes (SRBC), is administered together with SRBC. This results in more than 99% suppression of the IgM response. We are now investigating the suppressive effect of IgG on IgG responses and the development of germinal centers in the spleen. In collaboration with Frida Henningson and Sergio Estrada, we are investigating whether IgG changes the distribution of SRBC in a living mouse using small animal PET scanning. The localization of SRBC in spleens will also be studied using confocal microscopy.

BIOLOGICAL ROLES OF CD23, THE LOW AFFINITY RECEPTOR FOR IgE

Frida Henningson Johnson

We are investigating the biological role of CD23, the low affinity receptor for IgE. IgE can bind cells via CD23 and we have previously showed that CD23 on B cells has a central role in transport of antigen in complex with IgE. Transport of antigen to correct lymphoid organs is essential for an immune response to start, and this is of interest during vaccinations. However, it is not desirable to inject IgE because it may cause anaphylactic shock. Therefore we seek to investigate the possibility to use antibodies to CD23 to direct the antigen to B cells for further transport to lymphoid tissue and induction of a robust immune response.

CD23 is a C-type lectin, binding carbohydrate structures. CD23 is the only member of the Fc-receptor family that has this structure. However, many receptors in the C-type lectin family are so called pattern recognition receptors, binding pathogen associated molecular structures. These receptors are important in the innate immunity towards microbes and viruses. We are seeking new ligands for the receptor CD23, hoping to show that it, in addition to its IgE-binding properties can function as a pattern recognition receptor, showing a new, unique connection between pathogen infection and allergies. Knowledge concerning the mechanisms behind IgE-mediated enhancement of an immune response has a big significance on the knowledge in basal immunology and allergies.

Members of the group 2012
Frida Henningson Johnson, Assistant Professor
Hui Xu, PhD Student

Project workers in the group 2012
Lenny van Mechelen

Publications 2010 to 2012
1. Henningsson F, Ding Z, Dahlin JS, Linkevicius M, Carlsson F, Grönvik KO, Hallgren J, Heyman B. IgE-mediated enhancement of $CD4^+$ T cell responses in mice requires antigen presentation by $CD11c^+$ cells and not by B cells. *PLos One 2011;6(7):e21760 Epub Jul 6.*
2. Rutemark C, Bergman A, Getahun A, Hallgren J, Henningsson F**,** Heyman B. Complement Receptors 1 and 2 in Murine Antibody Responses to IgM-complexed and Uncomplexed Sheep Erythrocytes. *PLoS One. 2012;7(7):e41968. Epub 2012 Jul 25.*

Agencies that support the work
Hesselmans Foundation, Bror Hjerpstedt´s Foundation, Lars Hierta Foundation

IS CD23 A PATTERN RECOGNITION RECEPTOR?

Hui Xu, Frida Henningson Johnson

Judging from the structure of CD23 we hypothesize that it can also function as a pattern recognition receptor. This project is aimed at finding ligands using both $CD23^+$ and $CD23^-$ mice as well as soluble, recombinant CD23. The project includes cloning and expression of recombinant CD23, both in soluble and membrane-bound form, with or without the fluorescent tag YFP. Potential ligands to be tested include bacterial and viral structures, as well as structures on apoptotic and/or necrotic cells. Detection of binding between CD23 and ligand is performed using flow cytometry and/or confocal microscopy. When new ligands are identified we will also investigate which cell type that needs to express CD23 and the *in vivo* consequences of this binding.

CAN ANTI-CD23 COUPLED ANTIGENS BE USED AS A VACCINE TOOL?

Lenny van Mechelen, Hui Xu, Frida Henningson Johnson

In this project we try to take advantage of the immune-enhancing properties of CD23 in vaccination, where the main goal is to get the antigen to the right location and induce the immune system to produce antibodies against it. We seek to investigate the possibility to conjugate anti-CD23 antibodies to different antigens. This has been described earlier with OVA and rabbit gamma globulin as antigen, and we will investigate if the same immune enhancement can be seen when using other antigens such as viral proteins. First, are setting up the method in our lab conjugating OVA to the anti-CD23 antibody B3B4 according to previous publications. When the B3B4-OVA-system is set up we will continue to investigate if it can be used to induce an antibody response towards other antigens, for example the mouse-adapted H1N1-influenza virus PR8.

MAST CELLS AND THEIR PROGENITORS IN ALLERGIC AIRWAY INFLAMMATION (ASTHMA) AND RESPIRATORY INFECTIONS

Jenny Hallgren Martinsson

Mast cells contribute to many features of allergic asthma and express the high affinity receptor for IgE, FcεRI. Cross-linking of FcεRI-bound IgE with specific antigen degranulate mast cells and release proinflammatory mediators such as tryptase and histamine. Mast cells mature in tissues from committed mast cell progenitors, which numbers can be estimated by clonal expansion assays. The mouse lung contains few mast cell progenitors, but allergic inflammation increases the numbers dramatically. The increase in mast cell progenitors leads to higher numbers of mature lung mast cells and resembles the mast cell hyperplasia that occurs in asthma patients. Other triggers of mast cell progenitor recruitment to lung remain unknown. We study the mechanisms behind the mast cell increment in the allergic lung and the role of mast cells in allergic asthma and respiratory infections.

Members of the group 2012:
Joakim Dahlin, PhD student
Jenny Hallgren Martinsson, assistant professor, group leader
Cecilia Söderberg, Post-doc
Annika Westin, Technician
Behdad Zarnegar, PhD student

Project worker during 2012
Maria Friberg

Publications from 2010 to 2012

1. Collington, S J., J. Hallgren, J., Pease, JE., Jones, TG., Rollins, BJ., Westwick, J., Austen, KF., Williams, TJ., Gurish, MF. and CL Weller. 2010. The role of the CCL2/CCR2 axis in mouse mast cell migration in vitro and in vivo. *J Immunol* 184:6114-6123.
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5. Rutemark, C., Bergman, A., Getahun, A., Hallgren, J., Henningsson, F. and B. Heyman. Complement receptors 1 and 2 in murine antibody responses to IgM-complexed and uncomplexed sheep erythrocytes. *PLoS One* 7(7):e41968, 2012.
6. Dahlin, JS., Feinstein, R., Cui, Y., Heyman, B. and J. Hallgren. CD11c+ cells are required for antigen-induced increase of mast cells in the lung. *J Immunol*, 189:3869-3877, 2012.

Agencies that support the work
The Swedish Research Council, Swedish Heart-lung Foundation, Malin and Lennart Philipson Foundation, Åke Wiberg Foundation, Bror Hjerpstedt Foundation, Magnus Bergvall Foundation, Konsul ThC Bergh's Foundation, Ellen, Walter & Lennart Hesselmans Foundation, Agnes & Mac Rudberg Foundation, Uppsala University.

DEFINING MAST CELL PROGENITORS USING FLOW CYTOMETRY

Joakim Dahlin, Jenny Hallgren Martinsson

This project is aimed at identifying and quantifying mast cell progenitors with flow cytometry instead of using the indirect clonal expansion assay. The development of mast cells from early progenitors to a committed mast cell progenitor has been studied previously with contradicting results. One study suggests that the commitment to the mast cell linage takes place in the bone marrow whereas another study suggests that the commitment takes place in peripheral tissues. We hypothesize that we will identify a committed mast cell progenitor population in blood. We are searching for a defined mast cell progenitor population that in the presence of the multipotential cytokine cocktail only will develop into mast cells.

ARE MAST CELLS INVOLVED IN THE EXACERBATIONS OF ALLERGIC ASTHMA SEEN AFTER RESPIRATORY VIRUS INFECTIONS?

Cecilia Söderberg, Behdad Zarnegar, Annika Westin, Kjell-Olov Grönvik, Jenny Hallgren Martinsson

We hypothesize that mast cells are involved in the exacerbations of allergic asthma seen after respiratory infections via a combination of mechanisms. 1) Virus-infections may stimulate pathways that lead to recruitment of mast cell progenitors, which in itself may force exacerbations upon allergen challenge since more mast cells will be activated. 2) Mast cells express pattern recognition receptors that may be triggered upon the viral infection. Hence, mast cells may release more mediators if they are activated by a combination of allergen that crosslinks the IgE on the FcεRI receptors and viral products that trigger pattern recognition receptors.

To explore the hypothesis that respiratory infections trigger the recruitment of mast cell progenitors to the lung, we are using a laboratory strain of influenza H1N1 adapted to mouse (PR8) as a model of respiratory virus infection. Preliminary results suggest that intranasal infection of BALB/c mice with PR8 induce recruitment of mast cell progenitors to the lung. Influenza infection may be recognized by several different pattern recognition receptors. To explore what mechanisms that underlay PR8-induced mast cell progenitor recruitment to the lung, agonists of these receptors are screened for their potential to induce mast cell progenitor recruitment. Identified receptors can then be blocked by use of receptor deficient mice and/or blocking antibodies. To determine if mast cells are involved in the virus-induced exacerbations of asthma, groups of mast cell deficient, wild type and mast cell deficient mice reconstituted with mast cells will be used. We will compare how these groups of mice will respond when subjected to a combination of PR8-infection and a mouse model of allergic asthma.

PROPHYLACTIC TREATMENT WITH IgY ANTIBODIES AGAINST INFLUENZA VIRUSES DOES NOT INTERFERE WITH THE NORMAL DEVELOPMENT OF ADAPTIVE IMMUNITY AGAINST INFLUENZA

Kjell-Olov Grönvik

Aim : To develop new forms of intranasal-oral immuno-therapy by using IgY antibodies to control seasonal and pandemic influenza.
Methods: Laying hens were immunized into the breast muscle with HPAI H5N1 inactivated influenza viruses emulsified in Freund's adjuvant. Starting two weeks after last injection eggs were collected and IgY antibodies in egg yolks were purified by extraction with super Q water. Mice were treated intranasally with IgY plus influenza viruses and were scored for loss of weight as a sign if disease.
Results: Prophylactic treatment with IgY antibodies protected mice against lethal infection of H5N1 avian influenza virus. Such IgY also blocked virus invasion by H1N1 PR8 influenza virus during *in vitro* and *in vivo* challenges, demonstrating cross protection against different strains of viruses.
Three months later mice initially protected by IgY antibodies, were challenged with a lethal dose of PR8 virus. No signs of disease were observed indicating the development of a protective adaptive immunity to influenza. *In vitro* challenge of T cells from these mice with APCs from infected lung rapidly induced strong production of pro-inflammatory cytokines associated with protective memory T cells.
Conclusions: The heterogeneity of the IgY response to viruses in chickens generates antibodies with broadly protective activity against influenza viruses that can be used to control acute influenza virus infection without interfering with the development of adaptive immunity due to quiescent infection or conventional vaccination.

STUDIES ON THE MEMORY IMMUNE RESPONSE AGAINST THE H1N1 PR8 INFLUENZA VIRUS IN MICE

Objectives: To study phenotype, function and localization of influenza virus antigen presenting cells and virus specific memory T cells.
Methods: Mice were infected intranasally with a sublethal dose of live H_1N_1 PR8 influenza virus and after three months protected animals were challenged with a lethal dose of the homologous virus. At 36 hrs p.i. virus RNA in lungs and lymphoid organs was detected by RT-PCR. Six months post challenge mice were sacrificed and lymphocytes were isolated. T cells were co-cultured *in vitro* with influenza antigen presenting cells, APCs, from syngeneic mice primary infected for 36 hrs with influenza virus. Cell proliferation was measured by ^{3}H-thymidine uptake and cytokine production was analyzed by immunoaffinity in a Gyros platform or by flow cytometry.
Results: After primary infection virus RNA was detected in lungs and mediastinal lymph nodes. On the contrary, challenge of protected mice with a lethal dose of virus showed no PCR signal but T cells positive for pro-inflammatory cytokines were rapidly detected in lung and spleen. Infected dendritic APCs of lungs induced poor proliferation but strong cytokine production of the memory T cells *in vitro*. The inhibited proliferation was enhanced by removal of $CD4^+CD25^+$ regulatory T cells.
Conclusions: Mice protected against influenza virus develop an antigen specific sterile immunity and $CD4^+CD25^+$ regulatory T cells. Also, different populations of airway

associated influenza infected APCs regulate the immune response of influenza specific memory T cells differently.

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MOLECULAR BACTERIOLOGY

Dan Andersson, Andrea Hinas, Diarmaid Hughes, Linus Sandegren, Göte Swedberg

The area of molecular bacteriology at IMBIM is made up of five independent research groups. Overall our research is aimed at understanding how pathogenic and commensal bacteria genetically adapt and evolve in response to various external (e.g. antimicrobial drugs and biocides) and internal (e.g. deleterious mutations) selective pressures. A particular focus is to understand which biological factors determine the rates and trajectories of adaptive evolution when bacteria are exposed to antibiotics. Using a combination of genetics, experimental evolution, biochemistry and mathematical modeling we have shown that mutation, recombination and horizontal gene transfer rates, fitness costs of resistance and strength of the selective pressure are the main determinants of how rapidly resistant bacteria emerge and spread in a population. The long-term goal of this research is to increase our understanding of fundamental evolutionary processes and how we can slow down the emergence of drug resistant microbes by rational choices of drug targets, antibiotic use patterns and drug dosing. Ultimately this will allow us to treat infectious diseases in a more efficient way.

MECHANISMS, RATES AND TRAJECTORIES OF BACTERIAL EVOLUTION

Dan Andersson

Our research addresses the mechanisms and dynamics of molecular evolution in bacteria and how factors such as the extent and type of genetic variation, strength of selection pressures, compensatory mutations and population dynamics affect the tempo and mode of adaptive evolution. Our research is focused on two different areas: A) analysis of the various genetic factors that affect genome stability and variability in bacteria and B) analysis of the factors that influence the dynamics of the evolution of antibiotic resistance development. We study these problems in several bacterial species (S. typhimurium, E. coli, S. aureus and M. tuberculosis) using a combination of methods, including experimental evolution, bacterial genetics, molecular biology, biochemistry, whole-genome sequencing and mathematical modeling.

A. Genome variability and stability. The long-term goal of this project is to examine the evolutionary and mechanistic factors that influence genome stability and variability and how these factors influence the tempo and mode of bacterial evolution. We use the bacteria Salmonella typhimurium and E. coli as model systems to: 1. Examine how the extent and type of genetic variation affects bacterial fitness and rates of adaptive evolution. 2. Examine the role of gene amplification in adaptive responses to antimicrobial drugs and in the evolution of novel genes. 3. Examine the mechanism, physiological effects and evolutionary constraints on deletion formation. 4. Examine the functional role of ribosomal proteins and the mechanisms by which their absence can be genetically compensated. 5. Examine the fitness effects and constraints on horizontal gene transfer. 6. Examine the distribution of fitness effects of random mutations in different types of proteins and its impact on adaptive evolution. 7. Examine the mechanisms by which synonymous mutations can affect bacterial fitness and growth rates.

B. Mechanisms and dynamics of the evolution of antibiotic resistance. The overall objective of this project is to understand how antibiotic resistance affects the fitness, virulence and transmission of various pathogenic bacteria (e.g. S. typhimurium, E. coli, S. aureus and M. tuberculosis) and which factors determine how rapidly resistance develops in bacterial populations. Our main aims are to: 1. Determine how various types of resistance mechanisms affect bacterial fitness and virulence. 2. Determine how bacteria can compensate for resistance-conferred fitness costs. 3. Examine the importance of genetic epistasis on the rate and trajectory of multi-drug-resistance development and compensatory evolution. 4. Examine the feasibility of reversion of resistance by determining if reduced antibiotic use in community settings may result in a reduced frequency of resistance. 5. Identify mechanisms that confer resistance to antimicrobial peptides and determine the impact of these mechanisms on bacterial fitness and virulence. 6. Examine how very low levels of antibiotics can enrich for and select de novo resistant mutants in various types of laboratory and natural environments.

Members of the group during 2012
Dan I. Andersson, Professor
Ulrika Lustig, Research engineer
Karin Hjort, Researcher

Herve Nicoloff, Postdoc
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PLASMID SELECTION IN THE PRESENCE OF SUB-MIC LEVELS OF ANTIMICROBIAL METALS

Lisa Albrecht

Plasmid carrying resistance genes to multiple antibiotics is a growing concern. It is of interest to elucidate the modes of transfer and maintenance of these plasmids in bacterial populations. Since metal resistance genes are sometimes also found together with antibiotic resistance genes on plasmids, it is possible that the use and presence of metals could co-select for antibiotic resistance. Examples of such metals are arsenic, silver and copper. Arsenic is readily found in the soil at various geographic locations whereas silver and copper are both employed as antimicrobials, especially in the health care setting. The focus of this project is on positive selection conferred by plasmid encoded metal resistance genes and how the presence of these genes may enable a plasmid to be maintained in a population that is continuously exposed to even very low levels of metals. Two plasmids are included: pUUH239.2 harboring arsenic, silver and copper resistance systems and pMG101 encoding a silver resistance system. The relative resistance levels of the two plasmids to the relevant metals have been characterized. The subsequent step involves studying the effects of sub-MIC concentrations of these metals and how growth in this environment affects the fitness of the strains and the selective advantage of the plasmid. The competitive differences in this setting will be measured in by Fluorescence Activated Cell Sorting (FACS) using the fluorescent tags blue fluorescent protein and yellow fluorescent protein for detection of the strains.

ANTIBIOTIC SELECTIVE PRESSURE AT SUB-MIC CONCENTRATIONS

Erik Gullberg

When bacterial populations are exposed to antibiotics, bacteria carrying mutations giving them a higher resistance to the antibiotic will have a selective advantage over sensitive

bacteria, despite the fitness costs these mutations often cause. Not all resistance mutations have a fitness cost, there are also cases where fitness neutral mutations confer a high level of antibiotic resistance. The use of antibiotics in human and veterinary medicine can cause contamination of external sites, and many environments like sewage plants, farm run-off water and lake water can contain low levels of antibiotic residues. This project investigates how low, sub-MIC levels of antibiotics cause selection for resistant mutants. By doing competition experiments where a defined mix of resistant and susceptible bacteria is grown at different concentrations of antibiotics, the level that provides enough selective pressure for the resistant bacteria to take over can be determined. Besides chromosomal resistance mutations or genes, we will investigate how low levels of antibiotics affect the selection, conjugation and maintenance of conjugative resistance plasmids in bacterial populations.

We also study the enrichment of *de novo* resistant mutants at sub-MIC concentrations of antibiotics and identify the resistance mutations of these mutants using whole genome sequencing. Reconstruction of the strains with the candidate mutations in an isogenic background will show what mutations or combination of mutations are responsible for the resistance and which fitness costs they confer.

A NEW METHOD FOR IDENTIFICATION OF ANTIBIOTIC RESISTANCE IN BACTERIA IN CLINICAL MICROBIOLOGY

Erik Gullberg

When a patient comes to a hospital with a urinary tract infection or another infection that could be caused by bacteria, a sample is sent for analysis to a clinical microbiological laboratory. To identify the bacterium and to determine the antibiotic resistance profile, several growth steps and tests must be performed, many of these requiring overnight incubations. Because of this, the response time back to the physician is 2-3 days, and if the patient has a severe infection they will usually have been started on a broad-spectrum antibiotic or the default antibiotic for the suspected type of infection. Not only does this lead to over-use of antibiotics in case of virus infections, but the patient might also be given an antibiotic that has no effect in case of infection with resistant bacteria.

In this project, a method based on a combination of genotypic and phenotypic screening is being developed. The sample is grown for a few hours in the presence or absence of different antibiotics, and a sensitive probing technique allows for the simultaneous detection of both the species of bacteria and the resistance pattern. Padlock probes, which are single stranded DNA molecules designed for circularization when bound to target DNA, are utilized. The padlock probes target species-specific regions of the 16S rRNA in the bacteria. The ligated circles are used as templates for so-called rolling circle amplification (RCA), which gives amplification of the target. This method is sensitive, fast, and probes can be designed against the relevant species for different kinds of infections, which means that contaminating bacteria such as skin normal flora will not interfere with the detection.

SYNTHETIC CRISPR SYSTEMS TARGETING RESISTANCE GENES

Robin Hagblom, Erik Gullberg

Horizontal gene transfer, HGT, is an important process in microbial evolution. In some cases, the transferred genes can provide the receiving bacteria with competitive advantages

such as antibiotic resistance but in many cases they can be costly or even lethal. A CRISPR array (Clustered Regularly Interspaced Short Palindromic Repeats) along with CRISPR associated (cas) genes comprise the CRISPR/cas system which is a form of bacterial adaptive immunity against mobile genetic elements such as conjugative plasmids and phages. Although shown to be of the *Escherichia coli* type, little is known of the CRISPR/cas system in *Salmonella typhimurium* LT2 so further characterization is necessary. Once better understood, the native *Salmonella* system could be reprogrammed to target a given mobile element. For example, extant clinical plasmids that carry antibiotic resistance genes can be targeted in the hopes of protecting bacteria from the uptake of such a plasmid and thus preventing the spread of antibiotic resistance in a given bacterial population.

IDENTIFICATION OF *DE NOVO* RESISTANT MUTANTS AT SUB-MIC CONCENTRATIONS OF COLISTIN.

Karin Hjort

Bacterial populations can increase their resistance and develop *de novo* resistant mutations against antibiotics at sub-MIC (minimal inhibitory concentration) levels. This can become a challenging environmental problem since sewage plants, lake water and farm run-off contains low levels of antibiotics. The measured amount for some antibiotics in these environments are in some instances within the range known to increase the frequency of pre-existing antibiotic resistant bacteria and generate antibiotic resistance *de novo*. This project is focused on the antibiotic colistin and its ability to enrich for *de novo* resistant mutants of *E. coli* and *Salmonella typhimurium* during cycling at sub-MIC levels of colistin. Colistin is clinically used for multiresistant bacteria such as extended-spectrum β-lactamases (ESBL) producing bacteria.

Our preliminary results indicate that resistant populations of *E. coli* and *S. typhimurium* are generated at sub-MIC concentrations of colistin and that the level of antibiotic resistance is increased during the cycling experiment. Whole genome sequencing will determine if the mutations that emerged at sub-MIC are targeting similar functions as high concentrations of colistin generate. These experiments will give us a better understanding of the spread of antibiotic resistance at sub-MIC levels of antibiotics in the environment.

ESTABLISHING OF AN *IN VIVO* METHOD FOR COMPETITION ASSAYS OF ANTIBIOTIC RESISTANT PHENOTYPES

Karin Hjort

Most experimental setups to study evolution and fitness cost of antibiotic resistant strains are *in vitro* experiments using defined media and antibiotic concentrations. The establishment of an *in vivo* model mimicking environments such as natural water and sewage plants will increase our understanding of fitness costs of antibiotic resistant populations. In these environments nutrients are limited, bacterial populations are competing and phages are wide spread which changes the generation time of bacterial populations and probably also the fitness cost of an antibiotic resistant mutant. In addition these environments can contain low concentrations of antibiotics from veterinary and

medical use that can increase antibiotic resistant levels and generate *de novo* antibiotic resistant mutants. Bacterial fitness cost can be analyzed with competition experiments between antibiotic resistant mutants and wild type bacteria. The changes of bacterial populations are determined with fluorescently labeled bacterial populations in a FACS instrument.

Our preliminary data indicates that wild type *E. coli* can grow with a reasonable generation time, and population size in modified (autoclaved and filtered) sewage water from the inlet of Kungsängens sewage plant. Competition experiments in the modified sewage water with antibiotic resistant mutants versus wild type bacteria will determine if the fitness cost of antibiotic resistant mutants are different from a defined medium. These results will increase our understanding of the development and spread of antibiotic resistance in the environment.

RAPID AND EFFICIENT COMPENSATION OF LOW-FITNESS MUTANTS

Michael Knopp

The emergence and spread of antibiotic resistances has lead to the loss of many therapeutic options and represents a major public health concern. The molecular mechanisms of resistances often impose severe fitness costs to the resistant bacterial clones. The success of resistance mechanisms is strongly dependent on their influence on growth and survival, and it is therefore of importance to understand which factors ameliorate the fitness burden and increase the stability of antibiotic resistance mechanisms. Using long-term evolution experiments we were able to minimize the associated fitness cost of several resistance-causing mutations to four clinically important antibiotics: ertapenem, meropenem, fosfomycin and colistin. The resistance mechanisms include alterations in transport functions and two-component systems. By periodical screening of growth rates and resistance levels we determined the rate of compensation. In addition, we measured the correlation between bacterial fitness and susceptibility to these antibiotics. Our results show that compensation of fitness costs is very rapid and efficient. While the compensation for constructed knockout strains with defects in porins was only partial, compensated fosfomycin resistant strains increased their growth rate above wild type levels. Additionally, some of the evolved strains were able to tolerate even higher concentrations of the corresponding antibiotic than the nonevolved parental strain.

CAUSES OF FITNESS COSTS SYNONYMOUS MUTATIONS

Anna Knöppel

Synonymous mutations are sometimes deleterious, although they do not change the sequence of the polypeptide. Fundamental selective forces seem to underlay the selected base composition but very little is know about what levels the main fitness constrains operate (*e.g.* mRNA secondary structures or translation effectiveness). To investigate the mechanistic causes of the fitness reduction I use 6 synonymous single random base pair substitutions in a non-essential ribosomal protein, S20, in *Salmonella typhimurium* that have previously been shown to confer high fitness costs during rapid growth in rich medium. 8 independent lineages out of each strain have been evolved until compensatory mutations appeared that ameliorate the fitness costs. Whole genome sequencing analysis

indicates that mutations in *fis*, *putA, rpoA*, *rpoD*, and *rpsT* (coding for S20), as well as large duplications all including *rpsT*, can compensate for the fitness costs of the synonymous mutations. We hypothesise that the synonymous mutations in *rpsT* lowers the S20 levels in the cells and that the loss of function mutations found in *fis* (activator of rRNA transcription) could be a compensation for toxic non-functional ribosomes formed that lacks S20. Downregulation of rRNA would bring the ratio rRNA:S20 closer to 1:1 in the cells. Accordingly, the duplications seen would increase S20 levels. Presently, the mutations found in the evolved strains are being reconstituted. I am also planning to compare S20 mRNA and protein levels for wild type and mutant S20 and to study ribosomal pausing patterns along wild type and mutant mRNA.

CONSTRAINTS ON HORIZONTAL GENE TRANSFER

Anna Knöppel

Horizontal gene transfer (HGT) plays a major role in bacterial evolution and is known to be a key mechanism in the spread of pathogenicity determinants and antibiotic resistance. Apart from mechanistic constrains, the fixation of HGT events is greatly determined by selection and fitness effects. In this project we experimentally test the causes of fitness constrains of HGT. Random DNA fragments of *Bacteroides fragilis*, *Proteus mirabilis* and human intestinal phage are being inserted into a neutral position of the *Salmonella typhimurium* chromosome and the fitness costs of the inserts are determined and correlated to size and origin of insert, GC content, gene type, and the expression level of inserts. The data suggests that a substantial portion of the inserts could be nearly neutral and, surprisingly, three inserts were found to confer a beneficial effect on fitness.

The high fraction of inserts with beneficial effects (3/100) raised the question if beneficial inserts are more frequent than earlier thought. Experiments are now being set up in order to screen for beneficial inserts within a large library of insertion mutants. $10^4 – 10^5$ random DNA fragments originating from *B. fragilis* and *P. mirabilis* will be inserted behind an inducible promoter in a pre-determined position of the *S. typhimurium* genome, using the site-specific recombination machinery of transposon Tn7. High throughput sequencing before and after evolving the strains together with wild type would reveal if any insertion mutants have been selected for or against in the population. A specially developed tag system will make the sequencing possible.

MECHANISMS OF TIGECYCLINE RESISTANCE IN *ESCHERICHIA COLI*

Marius Linkevičius

Glycylcyclines, a new class of tetracyclines, were introduced in 1990s. Tigecycline is the main representative of the class and it has been used in medical practice since 2005. It is active against multidrug resistant gram-positive bacteria like methicillin resistant *Staphylococcus aureus*, vancomycin resistant enterococci and gram-negative pathogens producing extended spectrum ß-lactamases. However, resistance against tigecycline has been recently reported. Overexpression of unspecific RND or MATE family transporters was suggested to be the reason for the resistance to tigecycline. This study focuses on determination of resistance mechanisms to tigecycline and the consequential fitness costs in significant human pathogenic bacterium *Escherichia coli*. Two main groups of tigecycline resistant *E. coli* mutants were identified from whole genome sequencing data.

Genes involved in LPS biosynthetic pathway were found in one group. It is likely that these mutations affect the uptake of tigecycline, though the actual influx mechanism is not fully elucidated. Another group of mutations was linked to bacterial efflux and its regulation. Some of these mutations are known to be present in strains of *Enterobacteriaceae*, clinically resistant to tigecycline. In addition, a substantial fitness cost of these mutations was observed from growth assays, as tigecycline resistant *E. coli* mutants grew 10 to 34 percent slower than the wild-type *E. coli* strain.

TIGECYCLINE RESISTANT TET PROTEINS

Marius Linkevičius

Tigecycline overcomes major resistance mechanisms that render previous two generations of tetracyclines non-usable. It is believed that due to its bulkier chemical structure, tigecycline is not transported out of the cell by Tet efflux pumps, as the transport proteins cannot recognise the antibiotic as a substrate. In addition, a higher tigecycline affinity to ribosome prevents its dissociation from the A site in the 30 S subunit even in the presence of Tet ribosomal protection proteins, which cause conformational changes in ribosome. We are interested in whether evolution of tigecycline resistance conferred by Tet proteins is possible. DNA sequence libraries of Tet efflux and ribosomal protection proteins have been generated using error-prone PCR. According to the pilot selection of tigecycline resistant mutants harbouring mutagenised Tet efflux proteins, it is possible to select protein variants, with increased minimal inhibitory concentration for tigecycline. Larger scale selection experiments are being run to identify the exact mutations in Tet proteins that are responsible for decreased susceptibility to tigecycline.

SALMONELLA MUTANTS RESISTANT TO ANTIMICROBIAL PEPTIDES

Hava Lofton

Antimicrobial Peptides (AMPs) are listed as promising new drug candidates. They are part of the innate immunity of all living organisms. Many have intense, broad-spectrum antimicrobial activities and have been shown to have many other immune system roles, such as chemotactic response and modification of the host gene expression. We set out to test bacterial resistance development to AMPs and the resulting effects (i.e. fitness costs) in bacteria. By daily passaging small amounts of *Salmonella typhimurium* in periodically increasing concentrations of three different AMPs, CNY100HL, LL-37 and Wheat Germ Histones, we obtained mutants that tolerated much higher peptide exposure than parental strains. Whole genome sequencing identified several mutations, from which a subset was chosen for further investigation: *rfaY*, *pmrB* and *phoP* (all three genes are involved in modifying LPS) and were subsequently reconstituted in a wild type background. The mutation in *rfaY* (phosphorylates HepII in the LPS core) confers much of the resistance against all three AMPs. The fitness cost measured for all of the reconstituted mutants ranged from +0.8% to -16%. Significantly, the concentrations of AMPs used in our experiments are comparable to the levels found in human tissues, and that mutations such as *rfaY* could be selected de novo and possibly maintained by contact with host defense peptides though direct resistance and/or cross-resistance.

STUDY OF ANTIBIOTIC RESISTANT *E.COLI* IN DABBLING DUCKS (*Anas platyrhynchos)*

Ulrika Lustig och Marie Nykvist

It has been shown *in vitro* that very low concentrations of antibiotics, more than a hundred times lower than the minimal inhibitory concentration (sub MIC), can select for antibiotic resistant bacteria. Such low concentrations of antibiotics can be found in various natural environments. This leads to the question if resistance can be selected for in birds that are exposed to sub MIC concentrations of antibiotics in the environment, and if resistant bacterial strains can be spread long distances by migrating birds.
We have used four different ESBL (Extended Spectrum Beta Lactamase) *E. coli* strains isolated from gulls to infect a set of dabbling ducks. With this *in vivo* model we confirmed that dabbling ducks can be infected by gull ESBL *E. coli* strains. The infection persisted in some cases for four weeks, which allowed spread of resistant strains between ducks within the group during the experiment.

We have also studied how different concentrations of an antibiotic in the environment selects for antibiotic resistant bacteria using the *in vivo* model with dabbling ducks. The ducks were infected with an equal amount of two isogenic gull ESBL *E. coli* strains, one of them resistant to ciprofloxacin. During the study the ducks were exposed to concentrations ranging from 0,43-43 x MIC of ciprofloxacin in the drinking water. Resistant *E. coli* were selected for at a concentration of about 1xMIC in the water, corresponding to a much lower concentration within the duck.
This project is a collaboration between the groups of Josef Järhult and Björn Olsén (MedSci) and Linus Sandegren and Dan Andersson (IMBIM).

STRATEGIES FOR ANTIBIOTIC DEVELOPMENT TO REDUCE RESISTANCE

Ulrika Lustig and Cao Sha

Antibiotic resistance in clinical settings and the decline of antibiotic drug development is an increasing problem. The volume and pattern of antibiotic use influences the rate of resistance development and one idea is that dosing strategies in clinical settings can be optimized such as to minimize the emergence of antibiotic resistance while still maintaining efficacy. In order to collect *in vitro* data of bacterial growth rates and killing at different concentrations of antibiotics, we perform time-kill experiments on susceptible and well-characterized antibiotic resistant mutants of *E. coli*. By using the time killing data of MG1655 (a well-characterized laboratory wild type strain), an *in silico* model was developed. The model has been tested on 11 isogenic laboratory strains carrying mutations relevant to clinical ciprofloxacin-resistance. We also study how bacterial inoculum size, growth phase, and medium, affect the rate of bacterial killing by antibiotics. Ciprofloxacin time-kill experiments were also conducted on a set of clinical urinary tract infection (UTI) isolates. Data from these experiments were used to test the relevance of the extensive experiments on laboratory strains, and to further develop the *in silico* model. The *in silico* modeling is a tool to support predictions on how to dose one or several antibiotics in combination to optimize the effectiveness of therapy. The models can also be used to forecast the resistance potential of new drug candidates. This project is a collaboration

beetween the groups of Diarmaid Hughes and Dan Andersson (IMBIM), Lena Friberg and Mats Karlsson (FarmBio) and Otto Cars (Med Sci).

ROLE OF THE LON PROTEASE IN THE EVOLUTION OF INCREASED ANTIBIOTIC RESISTANCE

Hervé Nicoloff

Several studies revealed the frequent co-selection of *lon* mutations along with mutations causing low levels of antibiotic resistance. While *lon* mutations usually do not cause enough resistance for efficient growth on the selective media used, they can increase the frequency of isolation of mutants with higher resistance. Screening of clinical strains of *Escherichia* coli revealed that *lon* mutations could be involved in the development of resistance in clinically relevant pathogens. We hypothesized that *lon* mutations could be first step mutations in the evolutionary pathway to low antibiotic resistance. The focus of this study is to understand how *lon* mutations affect the development of resistance.

It was found that *lon* mutations caused a very small but consistent increased resistance to numerous antibiotics, a phenotype similar to that resulting from overexpression of the multidrug resistance pump AcrAB-TolC. This small resistance was caused by induction of *acrAB* by the transcriptional regulator MarA, which is stabilized in a Lon protease-deficient mutant. The increased frequency of selection of spontaneous mutants with higher resistance observed with a Δ*lon* strain depended on the small increase in resistance caused by the *lon* mutation itself. In those mutants, the higher resistance often resulted from spontaneous *acrAB* duplications combined with the increase in *acrAB* transcription caused by the *lon* mutation. This work revealed not only the nature and strict sequence of events in this evolutionary pathway (*lon* mutation followed by *acrAB* duplication), but also that the nature and frequency of the second mutations further increasing the resistance (e.g. *acrAB* duplication) was more important in the outcome of the selection than the effect that the first mutation had on resistance.

EVOLUTION OF NEW GENES THROUGH INNOVATION, AMPLIFICATION AND DIVERGENCE

Joakim Näsvall

It is a generally accepted idea that new genes can evolve from a duplication of an ancestral gene, freeing one copy from the constraints of purifying selection. Through random mutations and natural selection one of the copies may acquire a new function, while the other copy retains the original function. Several models for this process have been proposed, differing in the timing of the initial duplication, acquirement of the first beneficial mutation that leads to the new function, and the onset of selection. One model (Innovation, Amplification, Divergence; IAD) is based on the observation that many enzymes have weak secondary activities. A change in the environment, such as the presence of a toxic compound, a new nutrient or fixation of a deleterious mutation, can make such a minor activity beneficial, leading to a selective pressure to increase the activity.

We have developed a genetic model system to study the early stages of evolution of new genes. We isolated mutants in *hisA* that can partially substitute for *trpF* while still retaining some of the original activity. These bifunctional *hisA* alleles were placed on the

plasmid F'128 in a *Salmonella enterica* strain lacking the chromosomal *hisA* and *trpF* genes, and were allowed to evolve during serial passages in medium lacking both histidine and tryptophan. Amplifications started accumulating within the first few tens of generations, and dominated most lineages throughout the experiment. During 3,000 generations of continuous selection, some lineages accumulated additional mutations in *hisA*. In several of the lineages clones carrying two different *hisA* alleles within an amplified array appeared, each showing functional specialization towards one of the enzymatic activities. A follow-up to this study will determine the structure, stability, expression and specific activities of the evolved enzymes.

DOES CRYPTIC GENETIC VARIATION AFFECT EVOLVABILITY?

Joakim Näsvall

Neutral mutations, i.e. mutations that do not affect the original function of a gene, may affect later evolution of new functions by opening up new paths of evolution that are not available from the wild type sequence. The *hisA* gene will be placed under the control of a weak promoter so that the activity of the HisA enzyme is limiting for growth. Neutral mutations will be accumulated through rounds of mutagenesis and selection for maintained HisA activity. A collection of mutants containing one or several neutral mutations will be used as starting points for experimental evolution towards TrpF activity (see above).

FUNCTIONAL TRADE-OFFS DURING EVOLUTION OF NEW FUNCTIONS

Joakim Näsvall

Mutations that introduce or increase a new beneficial activity in a gene may have one of three different effects on the original activity: 1) the original activity is unaffected or only slightly decreased. 2) the original activity is lost proportionally to the gain in new activity. 3) the original activity is completely lost or severely decreased. The nature of the functional relationship between the new and original activity is likely to determine which paths evolution can take when both the new and old function is selected. To test which of the trade-offs are present we are setting up a model system based on mutations that confer TrpF activity to the *hisA* gene product (see above). The *hisA* gene will be mutagenized and variants with TrpF activity will be selected. The growth rates of strains carrying these mutant alleles in the absence of histidine or tryptophan will be used as a measurement of the different activities to determine which trade-off(s) exists in this system.

EXPLORING THE SUBSTRATE BINDING PROFILE OF A METALLO-ß-LACTAMASE AGAINST DIVERSE GROUPS OF ß -LACTAM ANTIBIOTICS

Song Sun

The extensive use and misuse of antibiotics during the last 60 years has led to the evolution and global spread of a variety of resistance mechanisms. Of high medical importance are ß-lactamases, a group of enzymes that can hydrolyze the ß-lactam ring present in all ß-lactam antibiotics. Metallo-ß-lactamases (MBLs) are particularly problematic due to their ability

to hydrolyze virtually all classes of ß-lactam antibiotics. A novel MBL (evMBL9) with low-level resistance against ß-lactam antibiotics was employed as the ancestral MBL during an evolution experiment to increase resistance. We designed and synthesized a mutant library in which the substrate binding profile was varied by randomizing six relevant amino acid residues. Mutants with increased resistance against seven different ß-lactam antibiotics (penicillin G, ampicillin, cefalotin, cefaclor, cefuroxime, cefoperazone and cefotaxime) were isolated and characterized. For most mutants, in spite of their significantly increased resistance, both evMBL9 mRNA and protein levels were strongly reduced (up to >20 fold), which indicated that the catalytic activities of these mutant MBLs were dramatically increased. Multivariate analysis showed that most mutants became "generalists" with increased resistance against most examined ß-lactams. The increased resistance and decreased protein level suggest that the improved hydrolysis in these novel MBLs is associated with decreased protein stability.

HOW PENICILLIN BINDING PROTEINS EVOLVE TO ß-LACTAMASES

Song Sun

Peptidoglycan is the major constituent of the cell wall. Penicillin binding proteins (PBPs) catalyze polymerization of the glycan strand and the cross-linking between glycan chains. The penicillin binding domain of PBPs are transpeptidases or carboxypeptidases involved in peptidoglycan metabolism. For both PBPs and ß-lactamases, the serine attacks the ß-lactam ring and forms a covalent acyl-enzyme complex. The deacylation step is very fast with ß-lactamases but extremely slow with PBPs. It has been postulated that ß-lactamases evolved from penicillin binding proteins based on structural data. The main aim of this project is to use experimental evolution as a tool to examine if it is possible to evolve a penicillin binding protein to a ß-lactamase under the selection of ß-lactam antibiotics. Fifteen genes encoding defined or putative penicillin binding proteins in *Salmonella typhimurium* LT2 genome were cloned in pUCBAD-kan and transformed into *Salmonella typhimurium* LT2 strain. The expression of the PBPs caused severe growth defect for most strains expect seven strains harboring the gene *ampH, dacA, dacB, dacC, pbpC, pbpG, ftsI* respectively. Seven libraries were constructed by random mutagenesis for each of the seven candidate PBP genes and in three libraries (*dacB, dacC, ftsI*) we were able to isolate mutants with increased resistance to penicillin G. Mutations were identified by sequencing the corresponding PBP genes in the mutants from the first round screening. Two *dacA* mutants and two *ftsI* mutants were subjected to a second round screening under an even stronger selection of penicillin G. These results indicate that PBPs has the potential to acquire weak ß-lactamase activity.

MECILLINAM RESISTANCE

Elisabeth Thulin

Many of the traditional antibiotics used for treatment of urinary tract infections (UTIs) have been rendered useless due to resistance development in the UTI-causing pathogens but resistance to mecillinam has remained low. Since fewer and fewer antibiotics are still effective it is important to understand how resistance arises to prolong their useful life time. The project focus on the genetics, physiology and evolution of mecillinam resistance,

examining mutants isolated in the laboratory as well as clinical isolates. This is achieved by identifying different mecillinam resistance mechanisms, how they arise and how they influence bacterial fitness and virulence. Mecillinam resistant mutants of *Salmonella typhimurium* and *Escherichia coli* have been selected in the lab. Identification of the resistance mutations was done for the *Salmonella* mutants and is ongoing for the *E. coli* mutants. Some compensatory mutants have been selected and will be sequenced. The mecillinam resistant clinical isolates were whole genome sequenced and compared to 20 reference strains (both clinical and isolated in the lab) to find which mutated genes are responsible for the clinical resistance. One mutation appeared in all mecillinam resistant strains. It contributes to some resistance, but it cannot alone explain high-level resistance. Since all clinical isolates have this low cost mutation it might be important as an intermediate in the development of higher-level resistance.

INTERCELLULAR RNA TRANSPORT IN THE NEMATODE CAENORHABDITIS ELEGANS

Andrea Hinas

RNA interference (RNAi), RNA-induced sequence-specific degradation of mRNA, has emerged as a major mechanism of gene regulation in most eukaryotes and has important implications in biomedical research and drug development. Extensive research has led to a relatively detailed understanding of this process in the short time since its discovery. However, a much less explored aspect of RNAi is the uptake and transport of RNAi silencing signals between cells in animals. Intriguingly, recent studies suggest that this represents a new means of cell-cell communication. Increased knowledge about spreading of RNAi is also of importance for the development of RNA-based drugs in order to efficiently deliver the drug to the location of the target mRNA. In some organisms, such as the nematode *Caenorhabditis elegans*, RNAi uptake and spreading occurs with high efficiency. By taking advantage of the many molecular and genetic tools available for *C. elegans,* a number of proteins required for RNA transport has been discovered.

Our current work is focused on elucidating the mechanism of function of the protein SID-5 in transport of RNAi silencing signals. To this end, we have performed a yeast-two-hybrid screen to identify SID-5-interacting proteins, which we have now started to analyze (see below). Methods currently used in our group include *in vivo* microscopy, genetics, transgenics, RNAi knockdown and immunofluorescence. Our research is expected to give clues about the natural roles for cell-cell transport of RNA in animals and may also aid in development of delivery methods for RNA-based drugs.

Members of the group 2012
Andrea Hinas (group leader)
Yani Zhao (PhD student)
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Publications 2010 to 2012
1. Hinas, A., Wright, A.J., and Hunter, C.P. (2012). SID-5 is an endosome-associated protein required for efficient systemic RNAi in *C. elegans*. Curr Biol *22*, 1938-1943.

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THE ROLE OF THE ENDOSOME-ASSOCIATED PROTEIN SID-5 IN TRANSPORT OF RNAI SILENCING SIGNALS

Yani Zhao, Benjamin Holmgren

From our previous studies, we know that SID-5 is required for cell-cell transport of RNAi silencing signals in *C. elegans* and that it localizes to late endosomes/multivesicular bodies (MVBs). Studies in *Drosophila* and mammalian cells show that MVBs are required for efficient cell autonomous RNAi whereas intact lysosomes seem to limit the RNAi efficiency. The co-localization of SID-5 and MVBs indicates that the RNAi transport pathway could also rely on endocytosis. However, the function of SID-5 in RNAi uptake and spreading is still unclear. We therefore performed a membrane yeast-two-hybrid screen to identify SID-5 interacting proteins. We identified several putative SID-5 interacting proteins including proteins with potential roles in endocytic pathways such as two SNARE proteins, a ubiquitin conjugation enzyme and an F-box protein, as well as proteins of unknown function. We are currently analyzing a number of these proteins further regarding co-localization with SID-5, mutant phenotypes etc. Together, these experiments will give us clues to how endolysosomal compartments regulate RNAi and transport of RNA between cells in animals.

BACTERIAL RESPONSES TO STRESS AND SELECTION

Diarmaid Hughes

Our main research interest is in bacterial genetics and evolution, specifically where it concerns the development of resistance to antibiotics and bacterial microevolution. In studying the important phenomenon of antibiotic resistance, an in-depth understanding of the selection processes and evolutionary principles behind fitness/resistance trade-offs is required for developing methodologies capable of suppressing the growth and spread of resistant bacteria. In this context we must look beyond just aiming for discovering new drugs or drug targets. It is critical to develop approaches to maintain and increase the therapeutic effectiveness of drugs already available.

We are studying the development of resistance to antimicrobial drugs, with a particular focus on the fluoroquinolones. Particular questions include how resistance development impacts on bacterial fitness in different environments and how bacteria respond to growth inhibition by compensatory evolution. The step-wise nature of antibiotic resistance evolution, and the co-evolution of resistance to multiple antibiotics are being studied. Antibiotic dosing strategies to minimize the risk of resistance development or selection are an important area of research where we collaborate with other groups within Uppsala University.

We also study bacterial microevolution in response to growth limitation caused either by genetic defects or physiological limitations imposed by the growth environment. These studies relate bacterial genetics and growth physiology with transcription, translation and gene expression regulation. Among the specific questions are the regulation of bacterial growth rate and ability to sense starvation, the importance of codon usage as a regulatory device and the possibility to evolve codon usage in response to demand. In addition we have a long-standing interest in bacterial mutation rates and the mechanisms and rates of gene conversion and genome rearrangements associated with repetitive sequences.

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BACTERIAL GROWTH REGULATION BY CODON USAGE BIAS

Jessica Bergman

The standard genetic code has 61 triplet sense codons encoding 20 amino acids. This leads to a redundancy and all codons are not used equally in all genes. During bacterial protein synthesis, the translation elongation factor EF-Tu brings tRNAs charged with amino acids to the ribosome. When bacterial cells are starved, there is a shift in the transcription pattern such that there is increased biosynthesis of amino acids and reduced production of ribosomes and ribosome-associated proteins, *e.g.* EF-Tu. Biosynthetic genes are usually not highly expressed during growth in rich medium and have a different codon usage pattern from very highly expressed genes such as *tuf*. Our hypothesis is that differences in codon usage act as a cue to signal the need for a shift in transcriptional pattern at the onset of starvation. A second element is the transcriptional regulator ppGpp, synthesised in response to starvation, and also involved in the shift in transcriptional pattern. EF-Tu is the most abundant protein in the bacterial cell and is encoded by two very highly expressed genes, *tufA* and *tufB* in *Salmonella enterica* serovar Typhimurium. We have tentatively identified codons that may act as attenuator sequences in the *tuf* genes during translation, working as a sensor for the nutritional status of the cell. Codon-specific pausing of the ribosome could lead to reduced EF-Tu production, inducing a stringent response with ppGpp production, and a rapid redirection of transcription towards biosynthesis of amino acids and other building blocks of the cell.

THE TRANSLATION ELONGATION FACTOR EF-Tu AND BACTERIAL GROWTH REGULATION

Jessica Bergman and Disa Hammarlöf

This project concerns growth regulation in *Salmonella enterica* serovar Typhimurium, with a focus on starvation responses. As a model system we are using a strain with a mutation in *tufA*, the gene for the translation elongation factor EF-Tu. This mutant EF-Tu is a weak binder of aminoacyl-tRNA. Bacteria that rely on a single, mutant *tuf* gene show an extreme slow growing phenotype. Using a combination of a screen for multi-copy suppressors of this slow growth, mutational studies, and biochemical assays, we discovered that the mutant bacteria overproduce the global transcriptional regulator ppGpp. This suggests that the mutant bacteria experience constant starvation even when they are grown in rich medium. During real starvation, ppGpp is produced and used to shift transcription towards biosynthetic operons and away from production of ribosomal proteins and ribosomal RNA.

This is also what happens in the slow growing *tuf* mutant, but instead of surviving real starvation it gets trapped in a vicious circle where the mutant bacteria produce less EF-Tu, experience reduced translation activity, and grow extremely slowly. By introducing genetic alterations affecting ppGpp production we can break this vicious circle and re-direct transcription activity so that it is more appropriate for a rich medium. This increases the levels of EF-Tu in the cell and also the probability of successful translation elongation, and improves growth rate.

EXTRAGENIC SUPPRESSORS OF RNase E TEMPERATURE-SENSITIVE MUTANTS

Disa Hammarlöf and Jessica Bergman

Why is the RNA processing enzyme RNase E essential? Bacterial cells need to process tRNA and rRNA and to degrade old or damaged mRNA transcripts in order to keep the transcription and translation machinery and processes in balance and attuned to growth requirements. In these processes, RNase E plays a central role, but the reason for its essentiality is unknown. Using a set of temperature-sensitive *rne* mutants in *Salmonella enterica* serovar Typhimurium, we selected and isolated extragenic suppressors that restored viability. Since these double mutants grow at the non-permissive temperature where mutant RNase E does not carry out its essential function, each of the suppressor mutations must somehow reduce the requirement for, or bypass, the essential function of RNase E. We mapped and identified a number of extragenic suppressors that are all related to translation or degradation of mRNA. Based of this we are exploring the hypothesis that the essential function of RNase E is to degrade mRNA, possibly to rescue ribosomes trapped on defective messages.

IMPORTANCE OF ACETATE UTILIZATION AND PROTEIN ACETYLATION FOR THE GROWTH OF MUTANT SUB-POPULATIONS ON AGING COLONIES OF *SALMONELLA ENTERICA*

Marie Wrande and Jessica Bergman

Spontaneous mutations in either *rpoB* or *rpoS* were found to confer a significant growth advantage on bacteria on aging wild-type colonies of *Salmonella enterica*. We have constructed a series of isogenic strains carrying mutations in *rpoB*, *rpoS* or both genes, and tested these for growth advantage in the context of colonies that are predominantly made of wild-type cells and find that the mutants have a very large growth advantage on aging wild-type colonies. We asked if acetate, which is excreted during exponential growth on rich medium and re-imported during the transition to stationary phase, played any role in the continued growth of these mutants. By combining the mutations in *rpoB* and *rpoS* with mutations in genes important for acetate utilization as a nutrient or for protein acetylation we are testing this hypothesis.

COMPENSATORY EVOLUTION AND RIFAMPICIN RESISTANCE

Gerrit Brandis

Multiple drug resistant *Mycobacterium tuberculosis* is causing serious problems in the treatment of tuberculosis. Many mutations in the β subunit of the RNA polymerase are

known to cause resistance to rifampicin, a first line drug against tuberculosis, but only little is known about the compensation of the fitness loss due to these mutations. In a previous study we have evolved a *Salmonella enterica* serovar Typhimurium strain harbouring a rifampicin resistance mutation, which causes a very strong fitness cost, to increased growth rate and compensatory mutations have been identified within the α-subunit, the β-subunit, and the β'-subunit of RNA polymerase. Due to its high fitness cost this particular mutation is not clinically relevant and has not been observed in clinical rifampicin resistant *M. tuberculosis* isolates. In the present study a *Salmonella* strain harbouring the rifampicin resistance mutation *rpoB* S531L, which is found in up to 75% of all clinical rifampicin resistance *M. tuberculosis* isolates, was evolved by serial passage to increased growth rate. Compensatory mutations have been identified in the same regions as previously observed, showing the importance of these types of compensatory mutations in clinical tuberculosis cases.

THE EFFECTS OF CODON USAGE BIAS ON BACTERIAL GROWTH RATE

Gerrit Brandis

All organisms utilize a universal genetic code in the translation of genetic information from messenger RNA into proteins. The genetic code is a triplet code with combinations of four different nucleotides. Thus, there are 64 different triplet combinations. These 61 sense codons encode only 20 different amino acids, which means that the genetic code is redundant. Despite the fact that the codon usage has no effect on the sequence of the translated protein bacteria have developed a strong bias towards the usage of certain codons over others. This codon bias is especially strong in highly expressed genes. The *tufA* and *tufB* genes, encoding for elongation factor EF-Tu, are among the most highly expressed genes in *Salmonella* and have an extreme codon usage bias. Growth rate and translational accuracy are very sensitive to changes in the concentration or activity of EF-Tu, which makes the *tuf* genes a perfect candidate to study the effects of codon usage. In this project the codon usage of the tuf genes in *Salmonella* will be altered to a less biased version. The physiological consequences of changing the codon usage will be assessed and the constructed strains will be experimentally evolved to improve fitness. Consequently fitness improving mutations will be determined and phenotypic effects of single mutations assessed. The results hopefully help to understand the physiological significance of codon usage bias in highly expressed genes and how rapidly codon usage bias evolves under selection.

AMELIORATION MECHANISMS OF THE COST OF PLASMID CARRIAGE IN *ESCHERICHIA COLI*

Eva Garmendia

The horizontal transfer of plasmids and other pieces of foreign DNA into naïve bacteria may impose a fitness cost that must be ameliorated by genetic changes. This type of evolutionary change is very relevant in medical microbiology where the acquisition of plasmids is associated with antibiotic resistance, and in biotechnology, where the introduction of expression vectors may disturb the physiological balance of the engineered strains. Previous work addressed the question of whether fitness cost and compensation are associated with plasmid acquisition and carriage. They found that the serial passage of *Escherichia coli* strains carrying classical drug-resistance plasmids reduced the cost of the

plasmid; furthermore, they were able to show that the serial passage was associated with evolution occurring on both the chromosome and the plasmid. This project aims to map and identify the specific alterations that had occurred in those strains using whole genome sequencing analysis, and if they are of interest, perform new evolution experiments to study by what mechanisms and at what rate the fitness compensation can be achieved.

IMPORTANCE OF LOCATION AND ORIENTATION OF HIGHLY EXPRESSED GENES IN BACTERIA

Eva Garmendia

One important feature of bacterial genomes is that the organization of genes on the chromosome is often highly skewed. Thus, highly expressed genes are often preferentially located close to the origin of replication and transcribed in the same direction as the DNA is replicated. The current hypothesis is that genomic-scale organization reflects selection pressure for maximum growth rate, by increasing the relative copy number of highly transcribed genes and minimizing the frequency of clashes between DNA and RNA polymerases. This study focuses on testing the significance of position and orientation of a gene whose product is directly liked to growth rate, the elongation factor EF-Tu. EF-Tu is, under normal circumstances, the single most abundant cytoplasmic protein in both *Escherichia coli* and *Salmonella typhimurium*. The gene encoding EF-Tu is normally present in two copies, equidistant from the origin of replication. By constructing strains with one gene at the normal position and the other in different locations around the chromosome (and also in both orientations) I will investigate the physiological consequences of this change, and if the growth rate varies systematically depending on those factors and how this relates to position and orientation. Evolution experiments will then address whether sub-optimal location and/or orientation can be compensated and by which mechanisms.

CO-EVOLUTION OF RESISTANCE TO ANTIBIOTICS

Franziska Pietsch and Douglas Huseby

E. coli is naturally sensitive to the fluoroquinolone drug ciprofloxacin. There is no single mutation or genetic resistance determinant known that can raise the level of resistance above the clinical breakpoint. Hence, resistance towards ciprofloxacin arises through several mutational steps. To study the development of resistance to ciprofloxacin, the laboratory strain MG1655 was cycled in progressively higher concentrations of ciprofloxacin. We found that this selection for resistance to the fluoroquinolone was associated with the co-selection of resistance to rifampicin, an unrelated drug. Rifampicin targets RNA polymerase (RNAP) where single mutations in *rpoB* (coding for the β-subunit of RNAP) can lead to high-level resistance to rifampicin. In six out of eleven independent lineages, which were evolved in ciprofloxacin, we identified mutations in genes coding for RNAP. To study the genetic evolution of resistance, a number of end-point strains were analyzed by whole genome sequencing and clones of earlier cycling steps were locally sequenced for the presence of mutations. Since rifampicin is used as a first-line drug against tuberculosis and increasingly complemented by the use of fluoroquinolones, it is important to understand the possible consequences of co-selection for resistance development and explore the mechanistic basis for co-selection. This is being done by genotype re-construction and de-convolution, coupled with competition experiments in

revelant selective environments. The aim is to understand the selective basis of the co-selection

FITNESS COSTS AND COMPENSATION IN FLUOROQUINOLONE RESISTANCE DEVELOPMENT

Lisa Praski and Douglas Huseby

Fluoroquinolones are synthetic broad-spectrum antibiotics frequently prescribed to treat UTIs, gastrointestinal infections, respiratory tract infections, sexually transmitted diseases and skin and bone related infections. Fluoroquinolone resistance is developed by mutations in the genes coding for DNA gyrase (*gyrA* and *gyrB*), topoisomerase IV (*parC* and *parE*) and transcriptional repressors (*marR* and *acrR*) affecting the level of the multidrug efflux pump AcrAB-TolC as well as plasmid-bourne genes (*qnr*) coding for molecules protecting fluoroquinolone targets. Most resistance mutations cause a reduction in fitness, often manifested as a decrease in growth rate. In the absence of a selection pressure from antibiotics, resistant, less fit bacteria need to adapt to survive. Development of such compensatory mutations can contribute to the stabilization of resistant bacteria in the population. In this project, spontaneous ciprofloxacin resistance mutations were selected from a wild type strain. Resistant mutants were evolved by serial passage to select for resistance and fitness compensatory mutations. All mutations were then identified by whole genome sequencing and are being analysed by re-construction into a wild type strain. Competition experiments with resistant and susceptible strains will be performed to measure differences in relative fitness. The aim is to develop a model of the order in which different mutations appear and to explain the selective forces driving the specific pathways of evolution.

OSMOTIC EFFECTS ON SMALL COLONY VARIANTS (SCV) OF STAPHYLOCOCCUS AUREUS

Cao Sha

SCVs of *S. aureus* are associated with reduced susceptibility to aminoglycosides and enhanced persistence in mammalian cells. In the previous work we showed that FusE SCVs comprised three sub-groups. All FusE SCVs have mutations in *rplF*. The FusE-hem and FusE-men sub-group carry in addition mutations in genes coding for hemin or menadione biosynthesis, respectively. When these SCVs were selected for faster growth mutants, in most cases, they acquired compensatory mutations (or reversions) in the *rplF*, *hem*, or *men* genes. However, in some exceptional cases the original SCV-mutations were retained unchanged even though the growth rate was significantly improved. By whole genome sequencing, we identified mutations in four different genes among these growth-compensated SCV mutants. Each of the four genes could be associated by database annotation with transport across the cell membrane, and some were predicted to affect the accumulation of osmoprotectants. The addition of osmoprotectant chemicals to the growth media significantly improved the growth rate of several of the SCV mutants. To confirm and test these results, a new collection of SCVs was selected for aminoglycoside resistance. This collection of mutants is currently undergoing testing for osmosensitivity-related phenotypes. Considering the function of osmoprotectants, we propose that many SCVs are hypersensitive to osmotic stress and have a different optimum to the wild-type

strain. These phenotypes could be relevant to the ability of SCVs to survive and grow in particular human microenvironments.

STRATEGIES FOR ANTIBIOTIC DEVELOPMENT TO REDUCE RESISTANCE

Ulrika Lustig and Cao Sha

Antibiotic resistance in clinical settings and the decline of antibiotic drug development is an increasing problem. The volume and pattern of antibiotic use influences the rate of resistance development and the idea is that dosing strategies in clinical settings can be optimized such as to minimize the emergence of antibiotic resistance while still maintaining efficacy. In order to collect *in vitro* data of bacterial growth rates and killing at different concentrations of antibiotics, we perform time-kill experiments on susceptible and well-characterized antibiotic resistant mutants of *E. coli*. By using the time killing data of MG1655 (a well-characterized laboratory wild type strain), an *in silico* model was developed. The model has been tested on 11 isogenic laboratory strains carrying mutations relevant to clinical ciprofloxacin-resistance. We also study how bacterial inoculum size, growth phase, and medium, affect the rate of bacterial killing by antibiotics. Ciprofloxacin time-kill experiments were also conducted on a set of clinical urinary tract infection (UTI) isolates. Data from these experiments were used to test the relevance of the extensive experiments on laboratory strains, and to further develop the *in silico* model. The *in silico* modeling is a tool to support predictions on how to dose one or several antibiotics in combination to optimize the effectiveness of therapy. The models can also be used to forecast the resistance potential of new drug candidates. This project is made as a collaboration beetween the groups of Diarmaid Hughes and Dan Andersson (IMBIM), Lena Friberg and Mats Karlsson (FarmBio) and Otto Cars (Med Sci).

PERSISTENCE AND EVOLUTION OF PLASMID-BORNE ANTIBIOTIC RESISTANCE

Linus Sandegren

We study fundamental aspects of how resistance plasmids are maintained and disseminated between pathogenic bacteria and how they serve as platforms for evolution of antibiotic resistance. The main focus is to understand how factors such as stability, mobility, positive selection and fitness costs influence the evolutionary success of plasmids. We also examine how the bacterial "immunity mechanism" called CRISPR affects plasmid spread and maintenance and if it can be used to specifically target resistance genes and eliminate resistance plasmids from bacterial populations. The experimental systems used are based on clinically isolated multi-resistance plasmids encoding extended spectrum β-lactamases (ESBLs) in enteric bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) that pose an increasing clinical problem by providing bacteria with resistance to the most used antibiotics today, β-lactams such as penicillins and cephalosporins.

Four main themes are of particular interest in these studies:

1. What impact do low levels of antibiotics have on spread, selection and maintenance of multi-resistance plasmids?

2. What plasmid factors cause a fitness-cost on the host cell and can the fitness-cost of plasmid carriage be alleviated by the bacterium in the absence of antibiotics?

3. How common are gene amplifications during treatment and does the dynamics of gene amplification on plasmids accelerate evolution of new resistance genes?

4. Can CRISPR-based targeting of resistance genes be a way to eliminate resistance plasmids from a specific bacterial population?

From these studies we expect to gain new knowledge of how bacterial cells and plasmids co-evolve and how selection of new resistance can be accelerated through gene amplification and different antibiotic concentrations. Such knowledge can be used to design antibiotic treatment regimens that limit selection of resistance and minimize the potential for new resistance to evolve. We also aim to explore a novel system for targeted eradication of multi-resistance plasmids from defined bacterial populations without killing the bacteria (anti-resistance therapy). In the future such treatment might be a way to clear the resistance determinants from the bacterial flora of patients.

Group members 2012:
Linus Sandegren – Assistant professor
Marlen Adler – PhD student
Erik Gullberg – PhD student (se also Dan Andersson)
Marius Linkevičius – PhD student (se also Dan Andersson)

Visiting PhD student 2012:
Hasan Badrul – Uppsala University/Linnaeus University Kalmar

Project students 2012:
Mehreen Anjum
Mikael Sundell
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ESBLD EVOLUTION

Linus Sandegren

During 2005-2007 there was a large outbreak of a multi-resistant, ESBL-producing *Klebsiella pneumoniae* clone at the Uppsala University Hospital. We have been involved in the characterization of the outbreak both with respect to the bacterium and the resistance plasmid. Ongoing projects are dealing with further characterization of the outbreak clone and how the plasmid is transferred between different bacterial species, how it changes over time with different selective pressures and how it contributes to evolution of resistance against other antibiotics.

The multi-resistance phenotype of the *Klebsiella pneumoniae* that caused the outbreak at the Uppsala University Hospital was due to a large multi-resistance plasmid. We have determined the complete sequence of the plasmid using massive parallel sequencing. Analysis of the plasmid shows that it consists of a backbone that is highly similar to a previously sequenced Klebsiella plasmid but has a resistance cassette comprising 45-kbp that instead is highly similar to the resistance cassette from plasmids associated with *E. coli* belonging to the international outbreak lineage ST131. This combination of a backbone and a resistance cassette from different plasmids has occurred through direct homologous recombination, in part mediated through homology in shared mobile insertion sequences between the two plasmids. Furthermore, we find two regions on the plasmid matching chromosomal genes, one from *E. coli* and one from Ralstonia

spp., indicating that mobilization of genes from several different bacterial species has occurred. We have also detected conjugational transfer of the plasmid from the outbreak Klebsiella to *E. coli* of the patient's own intestinal microflora. However, the plasmid is only stable in Klebsiella with an increased loss-rate in *E. coli* and no further spread of the *E. coli* transconjugants could be detected during the outbreak. We are currently looking into how the plasmid has changed during the outbreak and what factors influences plasmid stability and loss/gain of genes on the plasmid.

STABILITY AND SELECTION OF RESISTANCE PLASMIDS AT VERY LOW LEVELS OF ANTIBIOTICS

Erik Gullberg, Mikael Sundell

The evolutionary success of a plasmid is largely determined by its potential to be stably maintained in the host population. Resistance plasmids are widespread among clinically important bacteria due to the beneficial resistance genes encoded on the plasmids. However, plasmids usually confer a fitness cost on the host cell under conditions when the beneficial factors encoded are not needed (i.e. when antibiotics are not present). Why plasmids pose a fitness cost on the bacterium is still unclear. Under such non-selected conditions plasmid-bearing cells will be at a disadvantage and loss of the plasmid will result in more fit segregants that may out-compete the plasmid-containing cells. Stable plasmid maintenance in a bacterial population can therefore only be achieved if the rate of plasmid loss (by segregational loss and/or fitness costs) is balanced by the rate of plasmid gain (by horizontal transfer and/or fitness advantages).

In this project we study the fundamental segregational stability properties of plasmids in different genetic backgrounds to understand why some plasmids are very stably inherited in one host even though they infer a fitness cost on the host while they are relatively unstable in a closely related host. We also measure how low antibiotic concentrations of different antibiotics, for which the plasmid gives resistance, that are needed to counter-select the fitness cost and balance the stability in the population.

EVOLUTION OF CARBAPENEM RESISTANCE

Marlen Adler, Mehreen Anjum

Carbapenems are the most potent class of β-lactams and often the last treatment option for extended-spectrum β-lactamase (ESBL) producing pathogens. The use of carbapenems has increased in response to the threat of ESBLs. In this project we study the mechanisms by which bacteria spontaneously can increase their tolerance to carbapenems, the involvement of β-lactamases in further resistance development and how different carbapenems and treatment regimens affect resistance development.

The main spontaneous cause of carbapenem resistance or increased tolerance in *E. coli* is through changes in the expression of outer membrane proteins OmpC and OmpF. Loss of expression or down-regulation of the expression of OmpC and F can occur by mutational change in several regulatory proteins. Whole genome sequencing analysis of carbapenem-selected strains showed that duplication and amplification of plasmid encoded β-lactamase genes appear frequently.

Our studies show that the selective pathways towards increased levels of resistance are different for different carbapenems. Also the final level of resistance to different carbapenems (minimum inhibitory concentration – MIC) differs for the same mutations.

However there is a clear cross-resistance seen for all mutations. Furthermore, the production of β-lactamases increases the carbapenem MIC of mutant bacteria and the level of this increase also differ between carbapenems. These findings are important when treatment regimens are decided in order to minimize selection for resistant mutants.

DYNAMICS OF ERTAPENEM RESISTANCE IN A TREATMENT MODEL

Marlen Adler

In collaboration with the Department of Medical Sciences at Uppsala University we have used an *in vitro* dynamic system to study the effect of ESBL-production in *E. coli* on treatment with ertapenem. This system allowed us to mimic free drug concentrations that are achieved in the blood during the course of conventional ertapenem treatment. We detected porin deficient mutants after 48 hours in 55% of the experiments with ESBL-producers, but no mutants were detected from non-ESBL producers. Our experiments also showed that doubling of the conventional ertapenem dose does not prevent enrichment of these mutants. These findings are alarming and additional measures to prolong the life span of ertapenem as clinically useful antibiotic need to be considered.

Among clinical isolates *Klebsiella pneumoniae* is more frequently associated with carbapenem resistance due to ESBL-production than *E. coli*, but not much is known about the resistance mechanism. We are working to elucidate this mechanism and are especially interested in differences that allow *K. pneumoniae's* frequent associations with nosocomial outbreaks. The differences in fitness cost of resistance between *K. pneumoniae* and *E. coli* will be studied. The outbreak of ESBL-producing *K. pneumoniae* at the Uppsala University Hospital also gives us the opportunity to study the evolution and spread of carbapenem-resistance in over 100 clonally related clinical isolates sampled over five years.

These studies may directly affect carbapenem treatment regimes and help to decrease resistance development.

THE ROLE OF AMPLIFICATION OF β-LACTAMASE GENES IN CARBAPENEM RESISTANCE

Marlen Adler, Mehreen Anjum

Gene duplication and amplification (GDA) is a very frequent mechanism of adaptation in bacteria and has been reported to contribute to antibiotic resistance. We have found that amplifications of plasmid encoded β-lactamases with trace catalytic activity against carbapenems are selected when cells are exposed to carbapenems and that the amplifications elevate the tolerance to the antibiotics. In this project we study the effect of high-level amplifications of β-lactamase genes from the resistance plasmid pUUH239.2 on meropenem and ertapenem resistance.

The strong selection pressure of meropenem quickly led to dramatically increased copy numbers of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ and high minimal inhibitory concentrations (MIC), whereas only slightly increased copy numbers were needed to achieve high-level ertapenem resistance. High-level amplification of $bla_{\text{CTX-M-15}}$ provides a good target for gain-of function mutations towards increased catalytic activity against carbapenems. Continuous selection at high antibiotic concentrations might select for these mutations. However, strains with highly increased β-lactamase gene copy numbers had markedly reduced growth rates.

We also attempt to explore the potential of increased carbapenemase activity of

$bla_{CTX\text{-}M\text{-}15}$ by *in vitro* mutagenesis. It could be possible to obtain mutants that gain carbapenemase activity while retaining the original activity for cefotaxime (generalist), or mutants that lose their cefotaxime activity (specialist). We assume it might be easier to develop activity against ertapenem, because overexpression of $bla_{CTX\text{-}M\text{-}15}$ increased the MIC of ertapenem more than that of meropenem, which could indicate a better basal catalytic activity for ertapenem.

EVOLUTION OF TIGECYCLINE RESISTANCE

Marius Linkevičius

Tigecycline is one of the very few new antibiotics that target Gram-negative bacteria. It is the first compound belonging to the glycylcyclines, a group specifically designed to circumvent the prevailing resistance mechanisms against tetracycline antibiotics. Very little is known about development of resistance against this new class of antibiotics but clinical resistance has been reported, mainly through efflux pumps. We are looking at how spontaneous tigecycline resistance develops in *E. coli* and also if the dominant and wide-spread plasmid mediated resistance mechanisms against tetracycline (specific efflux pumps or proteins that prevent binding of the antibiotic to the ribosome) can evolve to also provide resistance to tigecycline. We find that tetracycline pumps have a small activity against tigecycline and that gene amplifications resulting in increased expression of pumps alleviate this activity. Mutations affecting the bacterial outer cell membrane also contribute to increased tolerance to tigecycline.

SYNTHETIC CRISPR SYSTEMS TARTGETING RESISTANCE GENES

Robin Hagblom, Erik Gullberg

The rapid development of antibiotic resistance in bacteria is one of the most serious current threats to human health, and this resistance is often spread through the exchange of mobile genetic elements such as plasmids. A CRISPR array (Clustered Regularly Interspaced Short Palindromic Repeats) along with CRISPR associated (cas) genes comprise the CRISPR/cas system which is a form of bacterial adaptive immunity against mobile genetic elements such as conjugative plasmids and phages. The capacity of the CRISPR system to specifically and efficiently degrade DNA solely based on the spacer sequences can be used to inactivate any unwanted genetic element. Could this adaptive immune system of bacteria be reprogrammed to target antibiotic resistance genes? In this study, clinical plasmids such as the pUUH239.2 will be targeted using synthetic CRISPR arrays in the hopes of protecting bacteria from the uptake of such a plasmid and thus preventing the spread of antibiotic resistance in a given bacterial population.

STUDY OF ANTIBIOTIC RESISTANT *E. COLI* IN DABBLING DUCKS (ANAS PLATYRHYNCHOS)

Ulrika Lustig, Marie Nykvist

It has been shown *in vitro* that very low concentrations of antibiotics, less than a hundred times lower than the minimal inhibitory concentration (sub MIC), can select for antibiotic resistant bacteria (Gullberg et al). Such low concentrations of antibiotics can be found in the environment e g in rivers and downstream sewage outlets. This leads to the question if

resistance can be selected for in birds that are exposed to sub MIC concentrations of antibiotics in the environment, and if resistant bacterial strains can be spread long distances by migrating birds.

We have used four different ESBL (Extended Spectrum Beta Lactamase) *E. coli* strains isolated from gulls to infect a set of dabbling ducks. With this *in vivo* model we could confirm that dabbling ducks can be infected by gull ESBL *E. coli* strains. The infection persisted in some cases for four weeks, which allow spreading of resistant strains by migration. The different gull ESBL strains were also transmitted between ducks within the group during the experiment.

We have also studied how different concentrations of an antibiotic in the environment selects for antibiotic resistant bacteria using the *in vivo* model with dabbling ducks. The ducks were infected with an equal amount of two isogenic gull ESBL *E. coli* strains, one of them resistant to ciprofloxacin. During the study the ducks were exposed to concentrations ranging from 0,43-43 x MIC of ciprofloxacin in the drinking water. Resistant *E. coli* were enriched in the ducks at a concentration of 0,86 x MIC in the water which corresponds to a much lower concentration within the duck.

This project is a collaboration between the groups of Josef Järhult and Björn Olsén (MedSci) and Linus Sandegren and Dan Andersson (IMBIM).

MUTATIONS AND GENETIC TRANSFER CONTRIBUTE TO EVOLUTION AND STABLE PERSISTENCE OF DRUG RESISTANT MICROORGANISMS

Göte Swedberg

Asexually reproducing microorganisms mainly rely on mutations for genetic variation. However, bacteria have evolved a variety of genetic transfer mechanisms that enhance genetic exchange and evolution of new traits like antibiotic resistance. My main interest has been sulfonamide drugs that act by inhibition of folate synthesis, thereby interfering with biosynthesis of nucleotides and some amino acids. Clinically, sulfonamides are used in very small amounts for treatment of bacterial infections, therefore sulfonamide resistant bacteria are not selected and are good models for stable persistence of drug resistance. In malaria treatment, sulfonamides are still very important for combination therapy. The rapid development of resistance to antimalarial drugs in Africa is a serious problem and we follow the development by field studies. One aim of the project is to explain the evolution of drug resistance on a molecular level and hopefully point the way towards design of better inhibitors both for bacterial and malarial infections. This is done by biochemical analysis of the target enzyme, dihydropteroate synthase, and the biochemical pathway, folate biosynthesis, where this enzyme has its function.

Members of the group during 2012
Göte Swedberg, associate professor
Nizar Enweji, PhD student
William Buwembo, PhD student (Makerere University, Kampala, Uganda), graduated Nov 28
Catherine Lwanira, PhD student (Makerere University, Kampala, Uganda)

Project workers during 2012
Salem Ali: *Bakterier som livnär sig på antibiotika*.
Lamija Hodzic: *Studiet av HPPK-DHPS som läkemedelstarget vid malaria*.
Ala Hussein Ali: *Genetisk kartläggning av plasmiden pLMO20.*
Peter Larsson: *Sökandet efter orsaken till trimetoprimresistens hos Streptococcus mutans.*
Caroline Aurosell: *Effects of internal deletions of hydroxymethylpteridine pyrophosphokinase-dihydropteroate synthase from Plasmodium falciparum.*
Sahil Aery: *In vitro evolution of dihydropteroate synthase: Effect of amino acid changes on enzyme function and development of resistance.*
Ahed Mardini: *Sulfa- Trimetoprimresistens mot kariesbakterier.*
Paul Saliba: *Erythromycin resistance acquired by horizontal gene transfer from the human microflora to the human pathogen Streptococcus pyogenes.*

International exchange during 2012
Catherine Lwanira, Makerere University, Uganda, worked in the lab May -June.
Göte Swedberg visited Makerere University, Uganda November 26-30.

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Agencies that support the work

SIDA/SAREC, Indevelop,

RESISTANCE TO ANTIFOLATE DRUGS IN TREATMENT OF MALARIA AND EVALUATION OF NEW DRUG TARGETS

Catherine Lwanira

Several antimalarial drugs act on the folate metabolism affecting synthesis of DNA precursors, especially dTTP. One example is Fansidar, a combination of pyrimethamine and sulfadoxine. This project involves further characterization of one already known drug target, the bifunctional enzyme HPPK-DHPS. An expression clone giving good amounts of bifunctional enzyme has been constructed. The plasmodial enzyme contains long stretches of amino acids that do not align with the corresponding bacterial enzymes. We are generating deletions in these stretches and have found both sequences that can be removed without losing enzyme activity as well as sequences that seem to be necessary for function. Another approach is to evaluate different treatment strategies by genotyping of malaria parasites exposed to antifolate drugs. This is done by PCR-based analysis of parasite DNA in blood samples from patients undergoing anti-malaria therapy. The project is based on collaboration with Makerere University, Kampala, Uganda and Bugando Medical College in Mwanza, Tanzania. Both countries are now switching from using antifolates to a drug combination with artemisinin and lumefantrine (coartem). The project is aimed at analysing the genetic changes in the parasites that results from this change in drug use. In vitro analysis of developing resistance to coartem are set up in Kampala and Mwanza, while improved methods for mutation detection are developed in Uppsala. The work during 2012 has mostly focused on methods for detection of emerging resistance to artemisinin compounds and disappearance of chloroquine resistance. The most remarkable

finding was evidence from Mwanza, Tanzania on reversal of chloroquine resistance, while samples collected in Iganga, Uganda showed high levels of resistance. No signs of artemisinin resistance were detected.

EVOLUTION OF DRUG RESISTANT *Plasmodium falciparum* IN EASTERN SUDAN

Nizar Enweji

The project addresses two related questions:
1) Are drug resistant *Plasmodium falciparum* genotypes in Sudan independent lineages or similar to those originally selected in south east Asia and later appearing in east and southern Africa?
2) Do mutant *P. falciparum* genotypes that persist during the lengthy dry and anti-malarial drug free period have lower fitness (ability to multiply and produce transmissible stages), compare to drug sensitive ones?

So far a number of patients with PCR positive samples from a full year has been recovered and they will form the basis for continued analysis of stability of parasite markers. Remarkable variation has been seen in samples collected during the dry season, showing that parasites in asymptomatic carriers are not just resting, but constantly growing without causing malaria symptoms. Some carriers show the same parasites each month, and our interpretation is that these individuals have just a single clone infection, while those showing variation have multiple clones. Most focus has been on investigating microsatellite markers, which are independent of drug pressure, but show the variety of parasite clones present in the area. With the help of these markers, we can see different patterns of clone fluctuation throughout the dry period in the asymptomatic carriers.

ESTABLISHMENT OF A DRUG-RESISTANT BACTERIAL FLORA IN HUMANS

William Buwembo

The use of antibacterial drugs exerts a selection pressure for drug resistant bacteria. Most important for the long term use of antibiotics is the impact of drug use on the establishment of drug resistant bacteria in the flora of commensal bacteria, that persist in the human body. Transfer of resistance determinants from commensal streptococci to *Streptococcus pyogenes* has been detected and one vehicle of transfer has been identified as a recently described defective conjugative transposon. In cases where we detect transfer, the defective transposon is inserted into a larger conjugative element, which is partly related to a transferable element found in *Streptococcus thermophilus,* but functions linked with excision and insertion of the element are identical to an elements found in *Streptococcus parasanguinis.* The genomic sequence of this bacterium is now available in GenBank, which has helped us to map the transfer.
William Buwembo has been investigating drug resistance in oral streptococci. Earlier he established that the majority of isolates of *Strep pneumoniae* and related viridans streptococci are resistant to sulfonamides and trimethoprim, and that the mechanism of resistance is in both cases mutational changes in the target enzymes dihydropteroate synthase and dihydrofolate reductase. In contrast, *Strep mutans* and *Strep sobrinus* from general dentistry patients show very low levels of resistance to these drugs. However,

HIV-positive patients taking co-trimoxazole prophylaxis have elevated levels of resistant isolates. A number of these have now been analysed and there are very few detectable changes in the target enzymes in resistant isolates, some variation in dihydropteroate synthase from *Strep mutans* has been seen, but not yet shown to be linked to resistance. We have now focused on these differences and analysing the influence of individual changes by site-directed mutagenesis. So far, we have not been able to pin-point the exact mechanism for either resistance, except in one strain where sulfonamide resistance is caused by mutational changes in the drug target enzyme.

MOLEUCULAR VIROLOGY/VIRAL MODEL SYSTEMS

Göran Akusjärvi, Tanel Punga, Catharina Svensson, Daniel Öberg and Göran Magnusson

Viruses are excellent model systems to study the basic processes of life, both in normal cells and under stress conditions and disease. Viruses typically encode for a limited number of key regulatory proteins that have a remarkable capacity to interfere and reprogram the normal control functions of the host. This interference with cellular processes sometimes goes haywire causing disease and under extreme conditions malignant transformation and/or death. To understand how viruses can have such a profound effect on regulatory networks at the cellular and organism level we are using medically important viruses to study basic mechanisms in gene expression, viral interaction with the innate immune response, the molecular mechanisms of viral latency, structure and function of virus-encoded microRNAs, function of viral oncogenes and mechanisms controlling the genetic stability of viruses. A thorough characterization of host-pathogen interactions is crucial to be able to understand the significance of the basic cellular processes of life for a virus survival and to design effective cures to viral diseases and/or develop novel strategies to use viruses in medical applications.

ADENOVIRUS IN BASIC AND MEDICAL RESEARCH

Göran Akusjärvi, Tanel Punga, Daniel Öberg

Viruses typically encode for a few potent regulatory proteins that have the capacity to rapidly and efficiently disarm host cell gene expression, resulting in a selective synthesis of virus specific gene products in the virus-infected cell. The great advantage with viruses is that they are small and therefore offer a simple genetic system that is easy to manipulate *in vitro*. Further, the mechanisms discovered in viral model system often recapitulates what life does in general. Thus, there have been, and still are, several lessons to be learned from studies of our viruses. Our current work is focused around several areas covering basic mechanisms in gene expression and medical applications of viral vectors.

We study:

- The remodeling of the host cell transcription and RNA splicing machinery during an adenovirus infection
- The function of the adenoviral L4-33K and L4-22K proteins in splicing and transcription
- The structure and function of adenoviral miRNAs
- Non-coding RNA mediated chromatin modification
- Function of exosomes in adenovirus infections
- Establishment and maintenance of long term persistent/latent infections
- Function of the histone like adenoviral core proteins
- Connection between Alu RNA expression and disease
- Novel functions of the adenoviral E1B oncoproteins
- Viral vectors in cancer therapy
- Epigenetic changes in Friedrich Ataxia

Members of the group during 2012
Göran Akusjärvi, professor, group leader
Göran Magnusson, professor emeritus
Feraz Ahsan, post doc (from March)
Anette Carlsson, technician
Sibel Ciftci, PhD student (from November)
Alexis Fuentes, researcher (until May)
Raviteja Inturi, research assistant (from July)
Wael Kamel, PhD student
Xin Lan, PhD student
Tanel Punga, assistant professor, group leader
Srinivas Thaduri, project assistant (until July)
Daniel Öberg, researcher, group leader
Sara Östberg, PhD student

Project workers during 2012
Tereza Brachtlova (from July)
Sibel Ciftci, PhD student (May-October)
Raviteja Inturi (until june)
Mohammad Nazmul Khan (until April)

Harini Sampath Kumar (until June)
Sandra Molen (2 months)
Julia Pickl (from October)
Maria Leonor Segurado Gouveia (2 months)
Casper Wahlund (from September)

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MAPPING OF THE DNA-PK AND PKA PHOSPHORYLATION SITES IN THE ADENOVIRUS L4-33K SPLICING ENHANCER PROTEIN

Mohammad Feraz Ahsan

The late genes of adenovirus are transcribed from the major late transcription unit (MLTU), giving rise to five different families of mRNA ranging from L1 to L5. The L4-33K protein is a virus encoded RNA splicing factor required to activate the early to late switch in adenovirus major late L1 alternative splicing. The L1-52,55K mRNA is produced both early and late after infection, whereas the L1-IIIa mRNA is restricted to the late phase of infection. The activation of splicing takes place in transcripts with a weak 3' splice site context. The L4-33K protein plays a key role in spliceosome assembly and is sufficient to convert nuclear extract prepared form uninfected HeLa cells to extracts with splicing properties almost identical to that of nuclear extracts prepared from infected cells.

It has also been shown that L4-33K specifically associates with the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) in uninfected and adenovirus-infected nuclear extracts. It is phosphorylated by DNA-PK *in vitro* in a double stranded DNA-independent manner and this phosphorylation has an inhibitory effect on the temporal switch in L1 alternative RNA splicing. L4-33K is also phosphorylated by protein kinase A (PKA), which has an enhancer effect on L1-IIIa splicing. Thus, it is interesting to observe that phosphorylations by two different protein kinases have opposite effects on the function of L4-33K as a splicing enhancer protein, suggesting a possible contributing DNA-PK and PKA as regulators of adenovirus alternative RNA splicing *in vivo*.

The L4-33K protein has a conserved region with an arginine and serine rich domain (RS-domain), which is critical for its splicing enhancer activity. This domain appears to be the target for the phosphorylation of the two regulatory protein kinases, DNA-PK and PKA.

MOLECULAR MECHANISMS AND EPIGENETIC REGULATION OF ADENOVIRUS GENOME STRUCTURE IN PERSISTENT INFECTION

Sibel Ciftci

Human adenoviruses (Ad) generally cause lytic infection in gastrointestinal tract, mucoepithelial cells in respiratory tract and in cornea. However, early evidences have shown that adenoviruses, subgroup C in particular, can also establish persistent infection mainly in lymphocytes of the human tonsils and adenoids. With this atypical life cycle of adenovirus, it enters to quiescent stage following the primary infection and can be maintained as episomal in its host cell for extended time periods. However, due to the lack of reliable cell systems, very little progress has been made on the molecular details in adenovirus persistent infection. Therefore, I have reconstituted Ad5 persistent infection in BJAB cells (B lymphocytes). With this system, I have been dissecting molecular mechanisms that may play significant roles during long-term infection of adenoviruses. Furthermore, to understand the long-term maintenance of adenovirus genome, the potential molecular mechanisms that might be involved in epigenetic signaling are also elucidated in this project. Indeed, the preliminary data have shown that adenovirus distinctly targets and regulates several cellular pathways and use different strategies in order to persist to B cells. My ongoing study also aims to enlighten the regulatory mechanisms of the viral and host cell chromatin-remodeling during the persistent infection. The ultimate goal of this project is to provide a better understanding towards adenovirus persistent infection and the molecular mechanism behind it.

ARE THE ADENOVIRUS VIRUS ASSOCIATED RNAs (VA RNAs) USED IN INTERCELLULAR COMMUNICATION?

Alexis Fuentes

Mammals with their adaptive immune systems posses at least two defense mechanisms that respond to dsRNA. Thus, long dsRNA (>50 bp) activates the interferon response pathway, whereas short dsRNA (<30 bp) activates the RNAi host antiviral defense mechanism. RNAi, is a well-documented antiviral defense mechanism in plants and insects but it is still unclear whether RNAi naturally limits viral infection in vertebrates. Viruses are master at implementing intricate strategies to impair the cellular defense mechanisms.

During the last decade convincing experiments have been presented showing that cell-to-cell communication is an important mechanism to traffic genetic information between cells. Possible routes include tunneling nanotubes (similar to bacterial conjugation), extracellular vesicles, apoptotic bodies, and nucleic acid-binding peptides. Among the most fashionable are extracellular vesicles for delivering cargo between cells, because these are easily secreted into the extracellular space and function as shuttles for the delivery of cargo between different cells within an organism.

Since adenovirus produces massive amounts of the VA RNAs, we have asked whether the VA RNAs functions as an extracellular messenger that spreads a signaling cascade to neighboring cells. Many reports have shown that endosomal derived exosomes act as vehicles for transferring nucleic acids and proteins from cell to cell. We have preliminary data showing that the VA RNAs can be found in exosomes prepared from infected cells. Our current work is focused at determining whether the exosomes prepared from infected cell cultures can regulate the innate immune response in neighboring cells.

CONSTRUCTION OF A VIABLE RECOMBINANT ADENOVIRUS INCAPABLE OF HIJACKING THE RNA INDUCED-SILENCING COMPLEX (RISC)

Wael Kamel, Anette Carlsson

VA RNAI is a 160 nucleotides long non-coding RNA, accumulating at high levels during the late phase of the viral infection cycle (approximately 10^8 molecules per cell). Adenovirus utilizes the VA RNAI molecule as a tool to silence the Interferon-induced Immune response by binding to dsRNA-activated protein kinase (PKR), This binding leads to PKR inactivation, thereby sustaining a high translational efficiency in late adenovirus-infected cells. On the other hand it suppresses the RNAi machinery in adenovirus-infected cells at three different levels. Firstly, it blocks the Exportin5 mediated nuclear export machinery, inhibiting the transport of per-microRNA and shRNA to the cytoplasm. Secondly it hijacks the Dicer enzyme acting as a competitive substrate and processing the VA RNAs into mivaRNAs. Finally these mivaRNAs saturates and blocks the RNA-Inducing Silencing Complexes (RISCs) with virus derived small RNAs. As a consequence of this multi-level inhibition of the RNAi machinery, the utilization of adenovirus as a therapeutic shRNA/siRNA expression vector has limitations in inducing RNAi in targeted cells, in addition to the off-target effects caused by expression of the mivaRNAs.

In order to study the function of the mivaRNAs and to find ways to overcome this problem, we have constructed recombinant Adenoviruses with a modified VA RNAI gene. In theory such mutated VARNAI genes will still be able to inhibit the PKR function and processed by the Dicer to mivaRNAI duplexes but these viral miRNAs will not be able to regulate their hypothetical target genes. Further, we are attempting to change the thermodynamic properties of the mivaRNAI duplex in order to block this duplex from loading into RISC. If this "decoy" VA RNA approach works this novel vector system can be tailored to become a standard virus used in gene therapy vector systems.

CHARACTERIZATION OF ADENOVIRUS L4-22K RESPONSIVE ELEMENTS IN THE MAJOR LATE PROMOTER

Xin Lan, Sara Östberg

Previously, our group has identified the late adenoviral protein L4-22K as an activator of transcription from the major late promoter (MLP), thus taking an active part in the early to late switch of adenovirus gene expression. The main responsive *cis*-element within the MLP is the so-called downstream element (DE), to which L4-22K binds and directs transcription of the MLP. The major activating element within the MLP seem to be the DE-element, but other elements are also shown to be activated *in vivo,* such as the upstream element (UPE) and the CAAT box. Recent data show that the DE and UPE are functionally redundant.

We have also identified an inhibitory element spanning the 5' splice site of the first tripartite leader downstream of the MLP initiator site. Mutations within this element enhances the L4-22K activated transcription from the MLP. *In vitro* transcription assays show that L4-22K induces the accumulation of 30-40 bp long pre-terminated RNA products. These results suggest that L4-22K can interact with this region thus blocking the transcription elongation from the MLP. Binding studies will be performed to confirm this interaction.

FUNCTIONAL CHARACTERIZATION OF THE ADENOVIRUS pVII PROTEIN

Raviteja Inturi

The adenovirus major core protein VII (VII) is a histone-like protein and is responsible for structural stability, functional organization and transcriptional regulation of viral DNA. It tightly complexes with DNA to form compact repeating structures termed ´adenosomes´ by analogy with the nucleosomes observed in nuclei of mammalian cells. Mature polypeptide VII (~19.4kDa) is synthesized from the precursor pVII (~21.8K) protein, by adenovirus protease proteolytic cleave during the final stage of virion maturation. The presence of precursor pVII and subsequent cleavage to form mature VII may be important for the functional and temporal regulation of adenovirus infection. As part of the study, we are characterizing the significant function of precursor pVII and mature pVII during a lytic adenovirus infection. We have identified specific residues of pVII and a cellular protein ubiquitin E3 ligase regulating the protein stability of pVII. Our results clearly indicated the differences in stability and localization between the precursor and mature pVII proteins. Our ongoing goals were to elucidate the molecular function of pVII and its cellular partners in adenovirus gene expression as well as their general role in eukaryotic gene transcription.

A LINK BETWEEN GEOGRAPHIC ATROPY (GA) AND AN ADENOVIRUS INFECTION?

Julia Pickl

Geographic Atrophy (GA) is an untreatable advanced form of age-related macular degeneration (AMD), a disease as prevalent as cancer in the industrialized world. It usually affects adults over the age of 50 years and causes vision loss or blindness. One molecular hallmark of GA is toxic Alu RNA accumulation within the retinal pigmented epithelium (RPE), which leads to their degeneration. Alu RNAs are encoded by Alu elements, which are the most abundant repetitive elements in the human genome (more than one million copies). They are specific to primates. It is supposed that they play a role in posttranscriptional regulation of gene expression.
As it was shown that adenovirus is able to induce Alu RNA transcription and to inactivate DICER1, which degrades Alu RNA, I examine the potential link between GA and an adenovirus infection.

DISECTING EPIGENETIC CHANGES IN NEURODEGENERATIVE DISEASE FRIEDREICH ATAXIA (FRDA)

Tanel Punga

Friedreich ataxia (FRDA) is a monogenic neurodegenerative disease caused by expanded GAA repeats in the frataxin (FXN) gene. The majority of FRDA patients (95%) have a pathogenic expansion of a trinucleotide GAA repeat within the first intron of the FXN gene. Generally, healthy individuals have up to 38 GAA repeats, whereas FRDA patients have most commonly 600-900 GAA triplets on both alleles of the FXN gene. The expanded GAA repeats correlate with a specific enrichment of repressive chromatin (heterochromatin)

within the first intron of the FXN gene. This particular epigenetic modification pattern correlates with reduced expression of the FXN protein, which has been considered as the underlying cause for FRDA.

My ongoing studies are focused on the interplay between different chromatin modifications and expanded GAA repeats on FXN locus. The ultimate aim of the project will be to specifically modify epigenetic pathways, which will allow the expression of the FXN protein in FRDA cells.

ELUCIDATION THE ROLE OF NUCLEAR MATRIX IN ADENOVIRUS INFECTED CELLS

Srinivas Thaduri

Nuclear matrix is the network of proteins found throughout the inside of a cell nucleus and is somewhat analogous to the cell cytoskeleton. However, in contrast to the cytoskeleton, the nuclear matrix has been proposed to be a highly dynamic structure. Thus, the nuclear matrix provides a structural framework for organizing chromatin, while facilitating transcription and replication. Small DNA viruses (SV40, adenovirus) have been shown to target nuclear matrix for their efficient DNA replication. We have recently identified two nuclear matrix proteins specifically interacting with the adenovirus structural proteins. In my ongoing studies I am characterizing the molecular function of these two proteins in adenovirus gene expression as well as their general role in host gene transcription.

THE ROLE OF EXOSOMES IN PERSISTENT ADENOVIRUS INFECTIONS

Casper Wahlund

Exosomes are a type of microvesicles, produced by most cells, transporting proteins and nucleic acids, and possibly playing important roles in both normal physiology and different states of disease. Exosomes were discovered some 30 years ago, and a common interest in the field increased greatly when some exosomes were found to have antigen-presenting capabilities. The past few years, it has been discovered that exosomes can carry both mRNA and micro RNA (miRNA) that can be delivered to, and elicit its functions, in target cells. It has also been discovered that the exosomal pathways can be used by pathogens in different ways, possibly to counter and/or downregulate immune responses and to facilitate spread of infection.

The aim of my project is to elucidate the role of exosomes during a persistent adenoviral infection. Our main hypothesis is that the exosomes are transporters of the noncoding adenovirus associated RNA (VA RNA). This transport of VA RNA, from infected to surrounding cells, could contribute to persistence of the infection. By extracting and analyzing the RNA from exosomes purified from persistently infected cells, conclusions about the role of exosomally transported VA RNAs can hopefully be made. The preliminary results are encouraging and suggests that only the Dicer processed forms of the VA RNAs, the so-called mivaRNAs, are efficiently captured in exosomes excreted from persistently infected BJAB cells (a human B cell line).

VIROTHERAPY AGAINST CANCER

Daniel Öberg

Adenovirus Onyx-015 was the pioneering agent in the field of cancer gene therapy using cancer selective vectors. This approach was based upon the fact that many tumour types have a dysfunctional p53 pathway. A deletion in the virus gene E1B, responsible to counteract the cellular p53 response to unscheduled DNA replication, was supposed to inhibit the virus in normal cells but allow its replication in cancer cells. As it turned out the viral E1B protein had several additional functions. This made the approach severely restricted in tumour targets and potency therein. The aim with my work is to decipher the intricate gene expression of adenovirus E1B in order to understand the additional functions of the E1B protein and thereby be able to engineer viruses that have enhanced tumour selectivity. As such my work consists of basic research with the possibility of clinical application.

THE FUNCTION OF L4-22K AND L4-33K PROTEINS EXPRESSED FROM DIFFERENT ADENOVIRAL SEROTYPES

Sara Östberg

Our group has previously shown that the closely related L4-22K and L4-33K proteins are integral parts of the early to late switch of adenovirus 5 gene expression. While the L4-22K protein enhances transcription from the major late promoter, L4-33K induces alternative RNA splicing of transcripts with a weak 3' splice site context. We are studying this by looking at the major late transcription unit 1 (L1). Early in infection only 52,55K mRNAs are produced from L1, but through an L4-33K mediated activation of a distal 3' splice site both 52,55K and IIIa mRNAs are accumulating in the late phase.

Both L4-22K and L4-33K are conserved in sequence between different adenovirus serotypes. We wanted to study whether the function of the two proteins also are conserved, and therefore set out to test the activity of the L4-22K and L4-33K proteins encoded by adenovirus serotypes 3, 4, 9, 11, 12 and 41 (representing different adenoviral subgroups) for activity in splicing and transcription. Our preliminary results suggest that while the function of the two proteins are conserved between most serotypes, there are some remarkable exceptions which will be investigated further.

ADENOVIRUS TYPE 12 INDUCED INTERFERON RESPONSE

Catharina Svensson

Adenovirus expresses a multitude of regulatory proteins to ensure efficient replication during a lytic virus infection. The immediate early E1A gene encodes two primary regulators, which are essential for transcriptional activation and forced entry of the host cell into the S-phase and for blocked induction of interferon (IFN) and IFN signalling. Despite many similarities, non-oncogenic and highly oncogenic adenovirus demonstrate differences in their productivity and cytopathic activity, where the highly oncogenic HAdV12 is less virulent and shows significantly less impact on host cell gene expression compared to the non-oncogenic HAdV2. We have shown that HAdV12 induces a specific activation of the IFN pathway during the later stage of infection. The inability of HAdV12 to completely evade the first line antiviral defence might explain the relatively low virulence of this virus compared to HAdV2, but an inadequate expropriation of the biosynthetic machinery of the host cell is also likely to play an important role. Preliminary results show a specific phosphorylation of PKR and a subsequent decrease in the accumulation of viral proteins. Our hypothesis is that the inability to counteract PKR activation is the result of poor expression of the HAd12V VA RNA.

Members of the group during 2012
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EFFICIENT PRODUCTION OF HUMAN ADENOVIRUS IN NORMAL MOUSE EPITHELIAL CELLS

Catharina Svensson and Staffan Johansson

The development of modified human adenovirus (HAdV) for oncotherapy is hampered by the lack of suitable immunocompetent mouse model systems where the oncolytic efficacies can be determined. The reason is that HAdV is severely defective for growth in rodent cells and although some viral gene expression occasionally has been detected, efficient production of new progeny virus has not been obtained. We have identified a non-transformed mouse cell line where the infection by HAdV2 is rapid and results in efficient production of new virus as determined by the ability of the recovered virus to superinfect standard human cell lines. Since there is a great interest for the possibility to use adenovirus vectors of less prevalence compared to type C HAdV, we have extended our analysis to members of all human HAdV types. Our results show that the here identified mouse cells also support growth of HAdV of types D and E, but not of types A, B or F. In this project we aim to determine the cellular prerequisite for multiplication of HAdV in mouse cells and also the molecular reason for the observed selectivity among HAdV types.

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DISSERTATIONS 2012

Beckman Sundh Ulla: Studies on phosphohistidine phosphatase 1, May 16, 2012

Eriksson, Jonas: Genetic and genomic studies in chicken. Assigning function to vertebrate genes, February 3, 2012

Huijbers, Else: Development of a cancer vaccine targeting tumor blood vessels, May 11, 2012

Jiang, Lin: Functional studies of the mechanisms of action of mutations affecting muscle growth in pigs and hair graying in horses, December 14, 2012

Noborn, Fredrik: Heparan Sulfate Dependent Mechanisms of Amyloidosis, March 23, 2012

Nordberg Niklas: Studies of budding yeast transcription factors acting downstream of nutrient signaling pathways, June 12, 2012

Olsson, Mia: Uncovering a novel pathway for autoinflammation - with a little help from a wrinkled friend, December 13, 2012

Ramachandra, Rashmi: Galactosaminoglycans Role in Brittlestar Limb Regeneration, September 18, 2012

Roy, Ananya: Mast cells as sentinels: Role of serglycin and mast cells proteases in infection and inflammation, August 17, 2012

Sun, Song: Dynamics and mechanisms of adaptive evolution in bacteria, June 5, 2012

Tranell, Anna: Regulation of HIV-a mRNA processing by cellular splicing factors, April 26, 2012

Zeller, Kathrin: Integrin signaling in cell adhesion and mechanotransdution: regulation of PI3K, AKT and ROS, April 27, 2012

LICENTIATE THESIS 2012

Femel, Julia: Tumor vascular markers - Function and therapeutic possibilities, June 7, 2012

PRIZES AND AWARDS 2012

1) Lennart Phlipson Award 2012

Jenny Hallgren Martinsson received the Lennart Philipson Award 2012.
In memory of Lennart Philipson the Board of Malin and Lennart Philipson Foundation donated a price that essentially consists of a grant for biomedical research with molecular orientation. Lennart Philipson award will provide support for promising young researchers who want to establish an independent research group for postdoctoral training.

2) Hilda and Alfred Eriksson prize from Royal Academy of Science

Leif Andersson - "*For his ground breaking studies in which he has used animal breeding that led to the understanding of the molecular mechanisms underlying many human diseases, among others, a type 2 diabetes* "

3) Wallenberg Scholars, The Knut and Alice Wallenberg Foundation

Kerstin Lindblad-Toh and **Leif Andersson** both received this prize.
'Wallenberg Scholars is a program designed to support and stimulate some of the most successful researchers at Swedish universities. The intention is to create a long-term security for the selected researchers to freely attack the big and difficult issues to reach new knowledge. Allocation is 15 million over five years and can be used freely in the manner researcher believes is most effective.'

4) The Limbic Prize (Limbiska priset in Swedish)

The students of the biomedical program awarded professor **Erik Fries** the Limbic Prize for 2012 for his great dedication as course leader of the Cellbiology and Biochemistry course and as chairman of the program committee.

"*Erik Fries has a pedagogical ability given to few, where as student you can leave the classroom and feel enlightened and informed. His way of structuring the lectures helps the students assimilate new knowledge. Erik is, in his role as program chairman, a great driving force for the improvement of the program and he puts great emphasis on the students' opinions.*"

5) Pedagogic prize from the medical students (Pedagogisk ros) to **Birgitta Tomkinson**.

6) Leif Andersson elected as a foreign associate of the National Academy of Sciences, USA

7) Dan Andersson elected fellow by the American Society of Microbiology, USA

8) Kerstin Lindblad-Toh elected member a member to the Swedish Royal Academy of Sciences.

9) Innovator of the year 2012 in Uppland by ALMI.

Leif Andersson together with Lisa Andersson and Gabriella Lindgren for their company CapiletGenetics AB, which has *developed an innovative diagnostic test which in a simple way using modern DNA technology can achieve significant resource savings in horse breeding by most likely to predict later performance horses. The company started is expected to have good commercial opportunities in a global mass market*

10) Göran Magnusson received the Gustaf Adolf medal in gold by Uppsala University for his long successful career as a researcher and lecturer at the Faculty of Medicine. In addition, he has held many positions such as Head of Department, Member of the Senate and the dean.

ECONOMY
(kSEK)

	2011	**2012**
Undergraduate Education Grant	24.779	24.325
Faculty Grant	51.563	64.954
External Grants	57.778	72.563
Others	48	684
Total	**134.168**	**162.526**

UNDERGRADUATE TEACHING AT IMBIM

IMBIM has about 18 full professors and associate professors, all of whom participate in undergraduate teaching. In addition there are about 6 assisstant professors and research fellows who also contribute to the teaching. Finally, there are about 40 PhD students who act as teaching assistants in the practical course work.

IMBIM participates in four different undergraduate study programmes: medicine, pharmacy, dispensing pharmacy, biomedicine and biomedical laboratory science. In all of these, laboratory work is an important part and IMBIM has about 600 m^2 dedicated to this purpose; some 20 different practicals on various subjects are given each year, some of which are common to two or three of the programmes. In addition, IMBIM manages a one year postgraduate programme allowing the students to rotate between different lab projects. Furthermore, IMBIM is in charge of a two year master programme in infection biology.

Medicine

In the medical programme, which is 11 semesters long, each course focuses on a specific medical topic - rather than the subject of a department - and the teachers come from different departments. Thus, teachers from IMBIM take part in courses covering topics like ”Energy and food stuff balance”, ”Homeostasis and endocrine regulation” and ”Attack and defense”. Teaching is done through regular lectures, study groups and practicals. The overall objective of these courses is to provide basic knowledge of the biological function of the human body and to create a basis for later clinical studies and future work in the medical profession. The major part of the Department´s contribution is in the field of metabolism and microbiology. Some 100 students are enrolled in this programme every semester.

Pharmacy

This 5-year programme leads to a Master of sciences in Pharmacy and is designed to prepare the students for work in retail and hospital pharmacies, pharmaceutical industry, government agencies and academic institutions. IMBIM is responsible for the teaching of microbiology. In this programme 90 students are enrolled every semester.

Dispensing pharmacy

This 3-year programme leads to a Dispensing Pharmacist Degree which prepares the students for work in retail and hospital pharmacies. IMBIM is responsible for the teaching of microbiology. Some 40 students are enrolled every semester.

Biomedicine

This 3-year programme aims to give students a sound understanding of the physiological and pathological processes occurring in humans. It contains different courses describing these processes from a molecular, cellular, genetical and medical perspective. Through practical sessions throughout the programme the students obtain experience in techniques used in current biomedical research. The programme aims at providing training for future activity in research, development and information. About 50 students are enrolled each year and the staff of IMBIM takes part in the teaching of biochemistry, cell biology and microbiology.

Biomedical Laboratory Sciences

This 3-year programme leads to a Bachelor of Medical Science (Major in Biomedical Laboratory Science) which prepares the students for work as biomedical scientists in diagnostic and research laboratories. Placements at external laboratories constitute a substantial part of the curriculum allowing the students to specilize within the programme. The major part of the Department's contribution to this programme is in the field of biochemistry. Some 40 students are enrolled in this programme every year.

POSTGRADUATE TEACHING

UGSBR
IMBIM manages a one year postgraduate programme allowing the students to rotate between different lab projects.

INFECTION BIOLOGY
IMBIM is in charge of a two year master programme in infection biology.

THE PhD PROGRAM AT IMBIM

During 2012 the department had 52 students registered for postgraduate studies. Twelve students defended their PhD theses and one student obtained a licentiate degree. New students are required to take a short introductory course in safety and general practice at the laboratory. In addition, the “older” PhD students take a great responsibility in helping the newcomers. Thus, the PhD students at IMBIM have formed an organization, the IMBIM PhD association board (IPhAB), which help new students with practical matter like help with employment, lodging and financial issues and good advice with what to expect from the department contra the responsibility of the student. IPhAB also organizes regular social events during the semesters to increase the interaction between students and employees at IMBIM. The department arranges several PhD courses that are aimed at broadening the knowledge of the research conducted at IMBIM. A monthly seminar series with the group leaders at IMBIM presenting their research area are arranged. After this lecture IPhAB arranges a social gathering with food to further stimulate interactions between PhD students and researchers working within the different disciplines at IMBIM. Further, weekly subgroup specific (Genomics, Biochemistry/Cell Biology and Microbiology) research seminars are arranged. These seminars give credit points in proportion to attendance. In summary, the PhD students have a collection of seminars within multiple disciplines to choose from which gives them a great opportunity to further expand their scientific expertise.

RESOURCE CENTRES AT IMBIM

CENTRE FOR COMPARATIVE DISEASE GENETICS AND GENOMICS
PI: Kerstin Lindblad-Toh
Co-PI: Leif Andersson (UU), Åke Hedhammar (SLU), Göran Andersson (SLU), Olle Kämpe (UU), Örjan Carlborg (SLU)

The aim of the center, funded by Formas, is to establish a world-leading Centre that uses domestic animals to study the genetics of multi-factorial disease. Genetic studies of domestic animals will provide insights into the molecular basis for phenotypic diversity and disease susceptibility that is difficult to obtain by studies in human or experimental organisms. The research focus will be on dogs, but we will exploit emerging opportunities in any domestic animal, such as chicken, pig and horse, as they appear. Four major disease areas will be covered within the center; 1) **Cancer**; We have performed genome wide association analysis on a large number of cancers including mammary tumours, osteosarcoma, lymphoma, hemangiosarcoma and mast cell tumours and regional resequencing of identified regions is ongoing. The candidate loci identify both previously known cancer genes but also new interesting pathways. 2) **Autoimmune and inflammatory disease**. 3) The **metabolic and cardiovascular disease** projects span a large set of disorders including models for muscle growth, diabetes and several cardiovascular projects in multiple species and 4) **neurological and behavioural disease** where we have demonstrated the potential of mapping neurological disease in dogs by identifying major genes for canine Amyotropic lateral sclerosis (ALS) and Obsessive Compulsive Disorder. For a number of the projects we are currently performing functional characterization of the susceptibility genes identified in the dog model as well as the role of the same genes in human patient samples.

PROTEOMICS RESOURCE CENTER

Åke Engström and Eva Andersson

This laboratory gives the scientific community an opportunity to at a low cost make use of recent developments in techniques and instrumentations for the analysis and identification of proteins. The facility for Expression Proteomics is equipped with 2-D electrophoresis systems, systems for post or pre-gel labeling of proteins, visible and UV light scanners, software for image analysis, spot picking systems, semi automated spot processing and MALDI-TOF/TOF instrumentation. The facility has expertise for 2-D analysis, mass spectrometry, image analysis, data base searches and general protein chemistry.

Our area of work is analysis and comparison of proteomes, identification of proteins in protein spots/bands by mass spectrometry, analysis of expressed proteins for quality control and analysis of proteins for post-translational modifications. The facility is open for all scales of problem solving or analysis, although the capacity for 2D gels might be a limiting factor for very large undertakings.

The service is primarily intended for identification of proteins from species with large numbers of genes or proteins characterized. The facility has in addition a limited capacity for de novo sequencing of proteins from any species. The service includes straightforward methods for characterization of expressed recombinant proteins. Considering the low cost for analysis this is highly recommended to avoid the potential risk of doing experiments with the "wrong" or modified protein. An MS analysis of intact expressed protein and a peptide mapping with MS give much better confidence than a simple SDS-gel analysis. If suitable for our techniques and knowledge we provide analysis of any type of sample

Organization

The expression proteomics facility is organized for running samples for researchers or for the researcher to use the equipment after approval. The latter is recommended for longer series of experiments. The facility is a part of the Proteomics platform at the Science for Life Laboratory in Uppsala.

Location

Equipment for handling and performing 2-D gel electrophoresis experiments and MALDI-Tof/Tof instrumentation are located at the Dept. of Medical Biochemistry and Microbiology, Biomedical Center (building D9 floor 3), Uppsala.

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UPPSALA GRADUATE SCHOOL IN BIOMEDICAL RESEARCH, UGSBR

Uppsala Graduate School in Biomedical Research (UGSBR) started in January 1997 as one of originally six local biomedical preparatory research schools initiated through support by the Foundation for Strategic Research (SSF). The school has as its prime objective to give university students, heading for a research education, a deeper knowledge about research and development, increasing possibilities to make active choices among the multitude of biomedical research fields and to establish productive national and international contacts.

As of Dec 2012, approximately 270 students have been accepted to UGSBR. The vast majority of UGSBR students have continued with PhD studies and of these, most have continued with their research carrier after receiving a PhD degree.

Management 2012:
Staffan Johansson, program director
Alexis Fuentes and Susanne Tingsborg, program administrators

SCIENCE FOR LIFE LABORATORY IN UPPSALA

SciLifeLab in Uppsala aims to be competitive on an international basis due to its combination of platforms for large-scale bioscience and application of these resources in front line research projects. The broad range of resources available, based on state-of-the-art technologies, have proven attractive both on a national and international level. The research performed at SciLifeLab has also received excellent rating in the universities' external research evaluations performed in 2011 at Uppsala University (Quality and Renewal 2011). We note in this context that for many research groups their connection to SciLifeLab is mentioned as a particular strength. The year of 2012 has been particularly productive for SciLifeLab Uppsala. There have been important achievements both for the infrastructure and in the research.

A growing research community
During 2012 SciLifeLab Uppsala has expanded substantially, both in laboratory space and people. The center now includes 800 persons, more than 150 of which staff the platforms. An important activity has been to recruit outstanding scientists to the research environment. This year we have recruited top-level researchers in important areas such as disease genetics, host-parasite relations for microorganisms, single cell genomics, bioinformatics, and RNA biology. In addition to the new research groups joining the center, a number of personnel have been recruited to strengthen the infrastructure activities.

Scientific resources
The technology platforms of SciLifeLab Uppsala have performed ~700 research projects, of which more than 30% were led by principal investigators from other universities than UU. This is a substantial increase over the ~ 500 projects conducted in 2011 and ~300 in 2010. Furthermore, the projects have increased in size, with expanding numbers of samples per project. Both the genomics and proteomics facilities have been used heavily during the year (>320 projects) and both with a substantial fraction of external users. The platforms have also devoted much effort to education on technologies offered by the platforms as well as subsequent data analysis. In addition, the technical platforms have performed technology development, resulting in a 26 scientific publications on methodology in 2012, as well as enabling novel insights in biology and medicine. Generally, the infrastructure has been developed in several ways during the year. New pilot platform facilities including a Single Cell facility, Biomaterial characterization and a Drug Optimization and Pharmaceutical Profiling Platform have been established at SciLifeLab Uppsala and the existing platforms have further developed the technologies they offer. Substantial external funding has enabled investments in new instrumentations and recruitment of personnel to the facilities. Since the platforms encounter an increasing demand from researchers and customers, much effort is put on simplifying the routines for using the resources. While the Uppsala platforms have been delivering on a national level already, they are preparing to meet the extended national responsibility that starts in 2013.

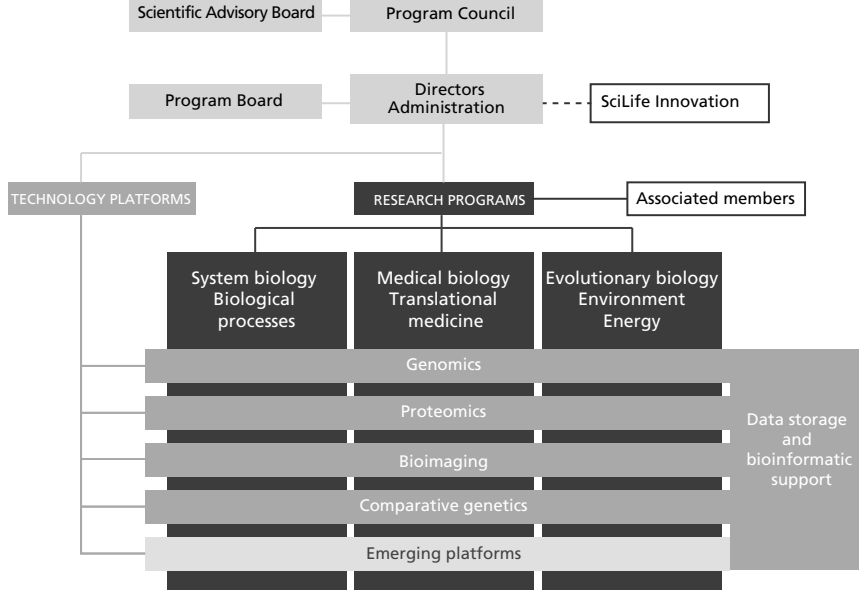
Collaborations
SciLifeLab has initiated discussions with several industry partners and other international networks to explore opportunities for collaborations. The AIM Day concept has been further expanded and utilized in 2012 with AIMDays in Cancer, Diabetes and Image resulting in >10 new collaborations.

A scientific community of high international quality

Besides the important mission to serve as an infrastructure, the ultimate goal for SciLifeLab is to generate excellent research results and new knowledge. SciLifeLab researchers perform research on an internationally high level. Research project performed at SciLifeLab Uppsala has resulted in >125 publications specifically citing SciLifeLab (43 published in high profile journals including Nature, Science and Nature Genetics) from within the SciLifeLab Uppsala community and many more from other users of the platforms. Highlights from these papers include investigations of human migration using genomic signatures (*Science*); detection of 100s of genomic regions important for the adaptation of stickleback to a fresh water environment (*Nature*), the mechanism by which transcription factors find and bind their chromosomal binding sites (*Science*), new mechanisms for killing cancer cells (*PNAS*), adaptation patterns for Atlantic herring (*PNAS*) and the wiring of the brain (*Nature Neuroscience*).

Overall, the published research covers a broad range of molecular bioscience. Considerable parts of the research is focusing on an increased molecular level understanding of human diseases, and of plants and microbes, finding new biomarkers for disease, and development of new treatments. By its nature the outcome of this research often depends crucially on technologies and methods for computational analysis performed within the center.

Fig. Organization SciLifeLab in Uppsala



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