DEPARTMENT OF
MEDICAL BIOCHEMISTRY
AND MICROBIOLOGY

ANNUAL REPORT
2014

Pictures taken by Katia Savary.

Differentiation therapy against glioblastoma multiforme

Glioblastoma multiforme stem-like cell cultures in spheroid form retain stem cell features: [bottom right] transmembrane protein CD133 (green) and transcription factor Sox2 (red) stain stem-like cells; [bottom left] actin (red) marks stem-like cells that differentiate and express intermediate filament protein GFAP (green) as spheroids transform to differentiated cells. These cultures help us define the molecular pathways that can be used to exterminate the stemness features using a so-called differentiation therapy.

Savary, K., Caglayan, D., Caja, L., Tzavlaki, K., Bin Nayeem, S., Bergström, T., Jiang, Y., Uhrbom, L., Forsberg-Nilsson, K., Westermark, B., Heldin, C.-H., Ferletta, M., and Moustakas, A. (2013) Snail depletes the tumorigenic potential of glioblastoma. *Oncogene* 32, 5409-5420.

García de Herreros, A. and Moustakas, A. (2014) Invasive cells follow Snail's slow and persistent pace. *Cell Cycle* 13, 2320-2321.

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INTRODUCTION

With the completion of the SciLife building (Navet) during late 2013 and the creation of of SciLife as an independent unit IMBIM underwent a major change at the sound of the New Years Bells. A change that most of you probably did not know happened. However, for me as the Head of the Department this change was noticeable. I personally expected to see a significant drop in the financial turnover and the number of personnel working at IMBIM. However, now looking back at 2014 my fears turned out to be largely unfounded. Although the granting situation has stiffened for many of us working in science there are also noticeable exceptions where scientists at IMBIM have received substantial support to continue their excellent work. For example, Matthew Webster received a large FORMAS grant to expand on his work on the evolution of the honeybee genome. Diarmiad Hughes who is involved in the large ENABLE project received a very generous grant to continue the development of new antibiotics. Last but not least, Kerstin Lindblad-Toh was selected in the first round of Distinguished Professors appointed by the Swedish Research Council. This prestigious position gives Kerstin a long-term (10 years) financial support to continue her successful work on comparative genetics and genomics. The number of persons working at IMBIM is also steadily increasing. All in all, IMBIM bounced back much quicker than I expected.

I would like to take the opportunity to welcome Gunnar Pejler and his group to IMBIM. During the year IMBIM initiated a recruitment of a new University Lecturer in Medical Biochemistry We had a whooping 29 applications to this position. Gunnar Pejler was ranked as number one and was therefore offered this position. With such a large and competent selection of applicants we took the chance to "kill two birds with one stone". Thus, Anna-Karin Olsson who came in second in this stiff competition was also offered a position as a University Lecturer in Medical Biochemistry. IMBIM likes to congratulate both Gunnar and Anna-Karin for their well-deserved appointments and expect them to do wonders as scientists and teachers during the coming years.

Two of our esteemed colleagues retired during 2014: Prof. Erik Fries and Prof. Kjell-Olov Grönvik. They have been asked to summarize their life in science in a short biography that is added as a new section to this year IMBIM-book. This section will be used in the future to commemorate the achievements of old colleagues and friends that goes into retirement.

Teaching:
Teaching of undergraduate and graduate students is a primary undertaking for IMBIM. During 2014 a total of 50 students were registered as PhD students and 8 students received their doctoral degree and 3 students a licentiate degree. The teachers at IMBIM does an excellent job something that is illustrated by the high grades that several of our courses have been given in various course evaluations. Further, Birgitta Tomkinsson received the most prestigious pedagogic prize awarded by the Uppsala University in 2014. The prize was awarded to Birgitta for her long time efforts to develop the "biomedicinska analytikerutbildningen" into a competitive educational program that is firmly based on state of the art scientific principles. IMBIM congratulates Birgitta for this well-deserved recognition and hopes that this will inspire her to even greater achievements.

Scientific Highlights:
The work at IMBIM has also been the focus in numerous press releases and newspaper articles describing the people and the research done at the Department. Much attention has been given to the new antibiotic research and animal genomics. This type of media attention is important to increase the public understanding of basic research and to improve the attitude towards science in general. During 2014 more than 105 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 I have picked out two examples of studies that illustrate the wide range of research carried out at our Department.

Matthew Websters group has conducted an analysis of genetic variation in populations of the honeybee Apis mellifera from all over the world by sequencing 140 individual bee genomes, providing insight into the evolutionary history and genetic basis of local adaptation in this species. This study provides a framework for future investigations into responses to pathogens and climate change in honeybees, something that is of hihest significance for this threatened species (Nature Genetics 46: 1081-1088)

Work in Dan Andersson and Linus Sandegrens groups have shown that very low levels of antibiotics and heavy metals can select for and maintain a costly plasmid that encodes resistance to these inhibitors. When several inhibitors were simultaneously present, the concentrations needed to maintain the plasmid were even lower. These findings suggest that the extremely low levels of antibiotics and heavy metals present in polluted external environments could contribute to the emergence and maintenance of antibiotic resistance in populations of pathogenic bacteria (mBio 5:e01918-14).

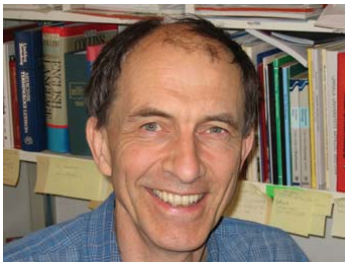
The end of the beginning:
The administrative and technical staff at IMBIM is of top-notch quality and does a marvelous job to support the research teams working at IMBIM. Without the effective support functions at IMBIM, the output in terms of research and teaching would rapidly deteriorate and the life for me as the head of the Department would become excruciating.

Finally, I would like to thank all of those who left IMBIM during the past year for your participation 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 take part in the future development of this Department.

Uppsala March 2015

Göran Akusjärvi
Head of the Department

Erik Fries – In and out of cells



With no particular career plan but with an interest in physics, I started my university studies in 1967 with mathematics followed by physics. However, the physics courses were not as interesting as I had hoped so I continued with general chemistry, physical chemistry and biochemistry. I then wanted to do more pratical work and was accepted as a PhD student at the Department of Biochemistry. My project concerned the release of proteins from membranes by detergent molecules. This project led me to a cooperation with a group at the European Molecular Biology Laboratory (EMBL) in Heidelberg that was studying the interaction of different detergents with a membrane-containing virus and the last year of my PhD studies I spent in their lab. After defending my thesis in May 1977, I was allowed to stay one more year as a postdoc at the EMBL and then began studying the initial interaction of the virus with its host cell. My analysis of the pH-dependence of the binding of the virus to different cells led us to the discovery of the process by which this and some other membrane bounded viruses infect cells: through internal pH-dependent fusion of viral and endosomal membranes.

In October 1978 I went to the laboratory of James Rothman at Stanford University for a second postdoc period. Rothman wanted to elucidate the molecular mechanisms underlying the vesicular transport of proteins from the endoplasmic reticulum (ER) to the cell surface. To achieve this goal he wanted me to reconstitute the transfer of a newly synthesized protein destined for the plasma membrane from the ER to the Golgi complex in a cell homogenate. However, this approach turned out to be unsuccessful, but I managed to obtain an inter-Golgi transfer in my cell free extract. Unexpectedly, this reaction became the basis for Rothman´s further work which earned him the Nobel Prize in 2014.

In November 1980 I returned to Uppsala to work on protein secretion with Prof Per Peterson at the Department of Cell Research. Here I was introduced to the use of primary cells in the form of hepatocytes isolated from rat liver. After about two years I got a position as an assistant professor at IMBIM. Here I continued my work on proteins secreted by hepatocytes, in particular how these proteins are modified during their transport to the cell surface. One of the proteins I studied was the chondroitin-sulphate containing protein bikunin, which, in a complicated way, is involved in the formation of the extracellular matrix. In 1990 I became associate professor and in 2000 full professor.

Kjell-Olov Grönvik - My life in science

In 1976 I was looking for a place to do my PhD studies and visited some different labs at BMC. When I came to Dept of immunology I had a very inspiring conversation with Hans Wigzell who was the first professor in Immunology at Uppsala University and at that time he was "chasing for" the antigen binding receptor of T lymphocytes, TcR, a hot topic at that time. The department had a very international composition of scientists from USA, Canada, Japan, Africa and eight different European countries working with B- and T-cell immunology and natural killer (NK) cells.

Professors Hans Wigzell and Jan Andersson were my supervisors and T cells became my research area and has mainly been so ever since. My PhD dissertation, "The role of T cell growth factor (TCGF) in the immune response", describes a T cell growth promoting and Natural Killer cell enhancing factor produced by activated T cells and was later termed interleukin 2 (IL-2). We were also able to immortalize that T cell function in T cell hybridomas after hybridization with a T cell lymphoma. During this period my supervisor Jan Andersson and I had a fruitful collaboration with the Basel institute for immunology which was the leading research lab in basic immunology in Europe at that time harboring the Nobel Prize Laureates in physiology or medicine Georges Köhler (1984) and Susumo Tonegawa (1987).

In 1983 I moved to Montreal in Canada for my post doc studies in Robert Murgita's lab at McGill University, where the main research was on pregnancy associated regulatory T and non-T lymphocytes present in lymphoid organs of pregnant mothers and neonatal mice. I brought the B-cell hybridoma technology from Uppsala and managed to produce monoclonal antibodies that recognized non-T suppressor cells and that upon administration interrupted pregnancy in mice. I was also involved in a project where infection with Neisseria meningitides was shown to selectively impair the function of B lymphocytes in mice. I obtained a position as Deputy assistant professor and was teaching immunology quite a lot at McGill. Two very happy years were spent in Montreal which is a city full of cultural events and fantastic restaurants. Thus I frequently visited Alexandre pub and restaurant downtown after working late in the lab and also found Alexandre's to be an excellent place for correcting student exams in a relaxing environment!

I was offered a tenure position at McGill University but my longing for Sweden and Uppsala made the decision easy for me to accept a position at Statens veterinärmedicinska anstalt, SVA, heading a newly formed laboratory for Immunology & Cell Culture where we mainly produced monoclonal antibodies for diagnostic use. In 1987 I was appointed docent in immunology at Dept of Immunology, UU, and six months later I was appointed Laborator at SVA. I resumed my research on T lymphocyte development in mice partly inspired by the cloning of the genes for TcR that was published by Professors Mark Davis and Pamela Bjorkman, USA, in 1988.

In a Graft-versus-Host (GvH) reaction in immunodeficient SCID mice my lab studied the development of grafted allogeneic and syngeneic T cells by analyzing the the TcR Vβ repertoire. We showed that acute severe GvH disease in these mice is associated with a

decrease of selective donor TcR Vβ specificities and an increased expression of pro-inflammatory interferon-γ which indicate that deletions of certain TcR specificities may disrupt the immunological homeostasis. Also, grafted $CD8^+$ T cells induced the formation of functional medullary thymic epithelium that supported the maturation of thymocytes into the $CD4^+8^+25^+$ stage which are otherwise not detected in SCID mice. In collaboration with labs in Buenos Aires and Montevideo my lab also studied the TcR repertoire in mice infected with the flagellate *Trypanosoma cruzi* that causes Chagas disease where the alimentary system, nervous system and muscle cells of the heart are infected. In collaboration with Professor Lennart Dencker the influence of the environmental poison tetrachlorodibenzo-p-dioxin (TCDD) on T cell development was studied and we showed that TCDD altered intrathymic T cell development and inhibited the activation of antigen specific T cells in mice.

I have been involved in many other collaborative studies on monoclonal antibodies and on T cell development but an example on a bit odd project was the nice collaboration with Professor Lars Bohlins group where we found that an extract from the red layer of the bark of *Alphitonia zizyphoides*, a rainforest tree found throughout the Pacific, enhanced the planting efficiency of a T cell hybridoma and monoclonal antibody production by a B cell hybridoma and the survival of bone marrow cells and normal B and T lymphocytes *in vitro*. Here the Swedish forest company SCA showed a genuine interest and funded the project. When enthusiastically outlining a pharmaceutical application of our findings and I was very surprised when SCA's Head of research said "It's nice to be able show that the bark is not only associated with the contamination of the environment but also contains beneficial factors that may improve our company's reputation among environmentalist". This taught me that scientific findings can have very different implications depending on the receiver of the message.

In 2005 I was appointed adjunct professor in immunology at IMBIM, UU, and during the last six years I have focused my research on antibody-based immunotherapy and the development of T cell memory against influenza A in an infectious mouse model. We have found that the immunotherapy does not interfere with the development of normal immunity and immunological memory against the influenza virus, and the strong T cell memory resides for the longest time in the regional mediastinal lymph nodes aligning the trachea. Furthermore, early and late phases after infection with influenza virus show different requirements of virus infected antigen presenting cells, APCs, in the recall responses of immune (memory) T cells and a compartmentalization of co-existing virus specific immunity and immune tolerance seem to develop in the peripheral lymphoid organs.

I find T cell immunity against influenza very exciting and I hope to be able to continue this research for some additional time since I'm a genuine "lab rat".

LIST OF CONTENTS

ADDRESS LIST

Department of Medical Biochemistry and Microbiology, IMBIM
www.imbim.uu.se

Address: Uppsala University, IMBIM, Box 582, 751 23 Uppsala, Sweden
Office: Phone +46 18 471 4444, Fax +46 18 471 4673

Adler Marlen	marlen.adler@imbim.uu.se
Akusjärvi Göran	goran.akusjarvi@imbim.uu.se
Albrecht Lisa	lisa.albrecht@imbim.uu.se
Algilany Kais	kais.algilany@imbim.uu.se
Andersson Dan	dan.andersson@imbim.uu.se
Andersson Eva	eva.andersson@imbim.uu.se
Andersson Leif	leif.andersson@imbim.uu.se
Annerén Cecilia	cecilia.anneren@imbim.uu.se
Ardesjö Lundgren Brita	brita.ardesjo.lundgren@imbim.uu.se
Arendt Maja Louise	maja-louise.arendt@imbim.uu.se
Axelsson Erik	erik.axelsson@imbim.uu.se
Barsh Sam	sam.barsh@imbim.uu.se
Bellomo Claudia	claudia.bellomo@imbim.uu.se
Berglund Jonas	jonas.berglund@imbim.uu.se
Bergman Jessica	jessica.bergman@imbim.uu.se
Bergquist Helen	helen.bergquist@imbim.uu.se
Bergström, Joakim	joakim.bergström@imbim.uu.se
Bianchi, Matteo	matteo.bianchi@imbim.uu.se
Bjerling Pernilla	pernilla.bjerling@imbim.uu.se
Bornelöv Susanne	susanne.bornelov@imbim.uu.se
Brandis Gerrit	gerrit.brandis@imbim.uu.se
Caja Puigsubira Laia	laia.caja@imbim.uu.se
Cao Sha	sha.cao@imbim.uu.se
Carlsson Anette	anette.carlsson@imbim.uu.se
Cedervall Jessica	jessica.cedervall@imbim.uu.se
Ciftci, Sibel	sibel.ciftci@imbim.uu.se
Dainat Jacques	Jacques.dainat@imbim.uu.se
Dierker Tabea	tabea.dierker@imbim.uu.se
Digre Andreas	andreas.digre@imbim.uu.se
Ding Zhoujie	zhoujie.ding@imbim.uu.se
Dogan Jakob	jakob.dogan@imbim.uu.se
Ek Pia	pia.ek@imbim.uu.se
Elvers Ingegerd	ingeger.elvers@imbim.uu.se
Engström Anna	anna.engstrom@imbim.uu.se
Engström Eva	eva.engstrom@imbim.uu.se
Engström Åke	ake.engstrom@imbim.uu.se
Enweji Nizar	nizar.enweji@imbim.uu.se
Eriksson Inger	inger.eriksson@imbim.uu.se
Farias Fabiana	fabiana.farias@imbim.uu.se

Femel Julia	julia.femel@imbim.uu.se
Filipek-Gorniok Beata	beata.filipek.gorniok@imbim.uu.se
Fries Erik	erik.fries@imbim.uu.se
Fuentes Alexis	alexis.fuentes@imbim.uu.se
Garmendia Eva	eva.garmendia@imbim.uu.se
Garoff Linnea	linnea.garoff@imbim.uu.se
Gottfridsson Eva	eva.gottfridsson@imbim.uu.se
Grabherr Manfred	manfred.grabherr@imbim.uu.se
Grahn Westin Annika	annika.westin@imbim.uu.se
Grönvik Kjell-Olov	kjell-olov.gronvik@imbim.uu.se
Gullberg Erik	erik.gullberg@imbim.uu.se
Gunnarsson Ulrika	ulgu@imbim.uu.se
Hallgren Martinsson Jenny	jenny.hallgren@imbim.uu.se
Hamilton Andrew	andrew.hamilton@imbim.uu.se
Hammar Veronica	veronica.hammar@imbim.uu.se
Heindryckx Femke	femke.heindryckx@imbim.uu.se
Heyman Birgitta	birgitta.heyman@imbim.uu.se
Hjort Karin	karin.hjort@imibm.uu.se
Hjälm Golovko Anna	anna.golovko@imbim.uu.se
Hoflund Tove	tove.hoflund@imbim.uu.se
Hughes Diarmaid	diarmaid.hughes@imbim.uu.se
Hultin Rosenberg Lina	lina.hultin-rosenberg@imbim.uu.se
Hultqvist Greta	greta.hultqvist@neuro.uu.se
Huseby Douglas	douglas.huseby@imbim.uu.se
Höppner Marc	marc.hoppner@imbim.uu.se
Imsland Freyja	freyja.imsland@imbim.uu.se
Inturi Ravi Teja	raviteja.inturi@imbim.uu.se
Jemth Per	per.jemth@imbim.uu.se
Jerlström-Hultqvist Jon	jon.jerlstromhultqvist@imbim.uu.se
Jern Patric	patric.jern@imbim.uu.se
Johansson Cecilia	cecilia.m.johansson@imbim.uu.se
Johansson Staffan	staffan.johansson@imbim.uu.se
Kamel Wael	wael.kamel@imbim.uu.se
Karlsson Andreas	andreas.karlsson@imbim.uu.se
Karlsson Åsa	asa.karlsson@imibm.uu.se
Kerje Susanne	susanne.kerje@imbim.uu.se
Kierczak Marcin	marcin.kierczak@imbim.uu.se
Kjellén Lena	lena.kjellen@imbim.uu.se
Knopp Michael	michael.knopp@imbim.uu.se
Knöppel Anna	anna.knoppel@imbim.uu.se
Kozyrev Sergey	sergey.kozyrev@imbim.uu.se
Källman Thomas	thomas.kallman@imbim.uu.se
Lamichhaney Sangeet	sangeet.lamichhaney@imbim.uu.se
Lan Xin ”Susan”	xin.lan@imbim.uu.se
Lantz Henrik	henrik.lantz@imbim.uu.se
Larsson Mårten	marten.larsson@imibm.uu.se
Li Jin-ping	jin-ping.li@imbim.uu.se
Lind Peter	peter.lind@imbim.uu.se
Lindahl Ulf	ulf.lindahl@imbim.uu.se
Lindberg Catharina	catharina.lindberg@imbim.uu.se

Lindblad-Toh Kerstin	kerstin.lindblad-toh@imbim.uu.se
Linkevičius Marius	marius.linkevicius@imbim.uu.se
Lofton Tomenius Hava	hava.lofton@imbim.uu.se
Lundgren Susanne	susanne.lundgren@imbim.uu.se
Lundin Erik	erik.lundin@imbim.uu.se
Lundkvist Åke	ake.lundkvist@imbim.uu.se
Lustig Ulrika	ulrika.lustig@imbim.uu.se
Magnusson Göran	goran.magnusson@imbim.uu.se
Maksimov Vladimir	vladimir.maksimov@imbim.uu.se
Malmqvist Mikael	mikael.malmqvist@imbim.uu.se
Mathioudaki Argyri “Iris”	a.iris.mathioudaki@imbim.uu.se
Meadows Jennifer	jennifer.meadows@imbim.uu.se
Moustakas Aristidis	aris.moustakas@imbim.uu.se
Murén Eva	eva.muren@imbim.uu.se
Naboulsi Rakan	rakan.naboulsi@imbim.uu.se
Namburi Ramesh	ramesh.b.namburi@imbim.uu.sr
Nicoloff Hervé	herve.nicoloff@imbim.uu.se
Nordin Jessika	jessika.nordin@imbim.uu.se
Nordli Olav	olav.nordli@imbim.uu.se
Norling Martin	martin.norling@imbim.uu.se
Nygren Kristiina	kristiina.nygren@imbim.uu.se
Nykvist Marie	marie.nykvist@imbim.uu.se
Näsvall Joakim	joakim.nasvall@imbim.uu.se
Olsson Anna-Karin	anna-karin.olsson@imbim.uu.se
Pettersson Jessica	jessica.pettersson@imibm.uu.se
Pettersson Mats	mats.petterson@imbim.uu.se
Pielberg Rosengren Gerli	gerli.pielberg@imbim.uu.se
Pietsch Franziska	franziska.pietsch@imbim.uu.se
Pijuan Galitó Sara	sara.pijuan@imbim.uu.se
Praski Alzrigat Lisa	lisa.praski.alzrigat@imbim.uu.se
Punga Tanel	tanel.punga@imbim.uu.se
Rafati Nima	nima.rafati@imbim.uu.se
Ramachandra Rashmi	rashmi.ramachandra@imbim.uu.se
Rajer Fredrika	fredrika.rajer@imbim.uu.se
Rask Lars	lars.rask@imbim.uu.se
Rask Malin	malin.rask@imbim.uu.se
Reyhani Vahid	vahid.reyhani@imbim.uu.se
Ringvall Maria	maria.ringvall@imbim.uu.se
Rodriguez Alejandro	alejandro.rodriguez@imbim.uu.se
Sörman Anna	anna.bergman@imbim.uu.se
Rubin Carl-Johan	carl-johan.rubin@imbim.uu.se
Rubin Kristofer	kristofer.rubin@imbim.uu.se
Saccoccia Fulvio	fulvio.saccoccia@imbim.uu.se
Salomonsson Maya	maya.salomonsson@imbim.uu.se
Sandegren Linus	linus.sandegren@imbim.uu.se
Schmid Martin	martin.schmid@imbim.uu.se
Scholz Veronica	veronica.scholz@imbim.uu.se
Shahidi Dadras Mahsa	mahsa.dadras@imbim.uu.se
Sköld Ola	ola.skold@imbim.uu.se
Spillmann Dorothe	dorothe.spillmann@imbim.uu.se

Steinhauf Daniel	daniel.steinhauf@imbim.uu.se
Strand Tanja	tanja.strand@imbim.uu.se
Strömbom Malin	malin.strombom@imbim.uu.se
Sung Song	song.sun@imbim.uu.se
Sundberg Christian	christian.sundberg@imbim.uu.se
Sundström Elisabeth	elisabeth.sundstrom@imbim.uu.se
Sundström Görel	gorel.sundstrom@imbim.uu.se
Svensson Catharina	catharina.svensson@imbim.uu.se
Svensson Mervi	mervi.svensson@imbim.uu.se
Swedberg Göte	gote.swedberg@imbim.uu.se
Sällman Almén Markus	markus.almen@imbim.uu.se
Söderhäll Carin	carin.soderhall@fackorg.uu.se
Tengvall Katarina	katarina.tengvall@imbim.uu.se
Thulin Elisabeth	elisabeth.thulin@imbim.uu.se
Tingsborg Susanne	susanne.tingsborg@imbim.uu.se
Tomkinson Birgitta	birgitta.tomkinson@imbim.uu.se
Tzavlaki Kalliopi	kalliopi.tzavlaki@imbim.uu.se
Wallberg Andreas	andreas.wallberg@imbim.uu.se
Wallerman Ola	ola.wallerman@imbim.uu.se
Webster Matthew	matthew.webster@imbim.uu.se
Wu Cheng Jun	chen.jun.wu@imbim.uu.se
Younis Shady	shady.younis@imbim.uu.se
Zamani Neda	neda.zamani@imbim.uu.se
Zarnegar Behdad	behdad.zarnegar@imbim.uu.se
Zetterqvist Örjan	orjan.zetterqvist@imbim.uu.se
Zhang Lu	lu.zhang@imbim.uu.se
Zhang Yanyu	yanyu.zhang@imbim.uu.se
Zhao Yani	yani.zhao@imbim.uu.se
Åberg Emma	emma.aberg@imbim.uu.se
Åkerblom Rehné	rehne.akerblom@imbim.uu.se
Öberg Daniel	daniel.oberg@imbim.uu.se
Östberg Sara	sara.ostberg@imbim.uu.se

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SCIENTIFIC PRESENTATIONS

COMPARATIVE GENETICS AND GENOMICS

Leif Andersson, Kerstin Lindblad-Toh, Pernilla Bjerling, Manfred Grabherr, Patric Jern, Gerli Rosengren-Pielberg, Matthew Webster, Jennifer Meadows, Erik Axelsson, Carl-Johan Rubin

Comparative genomics is of crucial importance to unravel gene function and regulation. We utilise specific human cohorts, domestic animals, natural populations and 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.

Genetic and genomic investigation of human cohorts. We have leveraged our knowledge of population genetics to delve into the genetics underpinning heritable disease with relevance to the health of Sweden. The specific population structure and bottlenecks of Sweden mean that smaller scale studies are likely to be able to reveal rare mutations or large effect on disease and at the same time allow for efficient burden analyses to be performed. Our current focus examines multiple diseases including the continuum of autoinflammatory - autoimmune diseases.

Genome evolution. We use comparative analysis to identify functional elements in the human genome and other 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. For example, we are investigating the newly identified human 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 get a deeper understanding of the molecular mechanism behind chromatin dynamics.

Retrovirus-host evolution. Retroviruses have colonized vertebrate hosts for millions of years, leaving traces in their genetic makeup as endogenous retroviruses (ERVs). This genomic ERV record provides a unique perspective on the long-term coevolution of retroviruses and their hosts. We use mainly bioinformatics to identify ERVs in genomic sequences of domestic animals and other vertebrate hosts to better understand retrovirus evolution and the effects of ERVs on host genome function and evolution.

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 AND NATURAL POPULATIONS

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 primarily using domestic animals models because domestication and animal breeding have caused major changes in many phenotypic traits. This gives an excellent 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's finches and Atlantic herring. The ultimate goal of the research is to identify the genes and mutations affecting a certain trait and thereafter to 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 2014

Leif Andersson, professor, group leader
Alvaro Martinez Barrio, post-doc (until March)
Sam Barsh, project assistant
Susanne Bornelöv, researcher
Stima Burri, project assistant
Chungang Feng, post-doc
Anna Hjälm Golovko, researcher
Rajesh Gupta, researcher (until June)
Ulrika Gunnarsson, researcher
Ulla Gustafson, technician (associated with Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences)
Freyja Imsland, PhD student
Susanne Kerje, researcher
Sangeet Lamichhaney, PhD student
Mårten Larsson, first research engineer
Khurram Maqbool, PhD student (until October; associated with Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences)
Rakan Naboulsi, PhD student
Jessica Pettersson, research engineer
Mats Pettersson, post-doc
Nima Rafati, PhD student
Doreen Schwochow, PhD student (associated with Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences)
Elisabeth Sundström, researcher
Ola Wallerman, post-doc (associated with Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences)
Shady Younis, PhD student

International exchange during 2014

Dr. Miguel Carneiro, University of Porto, Portugal (visiting researcher during one months)
Dr. Congying Chen, Jiangxi Agricultural University, Nanchang, China (visiting researcher during eight months)
Dr. Miriam Friedman-Einat, Agricultural Research Organization, Volcani Center, Israel (visiting researcher during 4 months)
Ms. Sara Negro, University of Cordoba, Spain (visiting student during three months)
Ms. Jinxiu Liu, China Agricultural University, China (visiting student during two months).

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

Rajesh Gupta, Mårten Larsson, Rakan Naboulsi, Elisabeth Sundström, Ola Wallerman, Shady Younis, 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 mouse C2C12 myoblast 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*. We have also shown that ZBED6 contributes to transcriptional regulation in pancreatic islet cells as well as human colorectal cancer cells. 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. 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 which now is available and which currently are undergoing careful phenotypic characterization.

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

DETECTING SIGNATURES OF SELECTION USING WHOLE GENOME RESEQUENCING

Alvaro Martinez Barrio, Sam Barsh, Susanne Bornelöv, Stina Burri, Miguel Carneiro, Congying Chen, Chungang Feng, Ulla Gustafson, Sangeet Lamichhaney, Khurram Maqbool, Jessica Pettersson, Mats Pettersson, Nima Rafati, 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 pooled samples to detect signatures of selection when comparing different populations that have 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 the detection of more than 7 million single nucleotide polymorphism and 38 loci with strong signatures of selection.

More recently we applied the approach to a comprehensive comparison if wild and domestic rabbits with the aim to reveal the genetic basis for domestication. The study is an advance in understanding animal domestication. We demonstrated that (i) rabbit domestication has a highly polygenic basis as signatures of selection was associated with a large number of genes, (ii) that changes in non-coding sequences have been more prominent than changes in coding sequences, (iii) that there were no example that gene inactivation played a prominent role, and (iv) that domestication were associated with changes in allele frequencies at many loci rather than fixed changes at a few major "domestication genes" with large effects.

Whole genome sequencing of individual birds have been applied to study the evolution of the Darwin's finches. We have sequenced 120 birds representing all species of Darwin's finches including the Cocos island finch, and two closely related tanager species. This has allowed us to (i) revise the phylogeny of this radiation, (ii) to demonstrate extensive interspecies gene flow during the evolution of these birds and (iii) to identify a gene controlling variation in beak shape within and between species of the Darwin's finches.

Another major research program concerns the Atlantic herring. The Atlantic herring is one of the most abundant vertebrates on earth and constitutes an enormous biomass in the North Atlantic and associated waters like the Baltic Sea. We have now made a genome assembly of the herring genome and have carried out whole genome resequencing of 20 population samples from the Baltic Sea, Kattegat, Skagerrak, North Sea, Atlantic Ocean and Pacific Ocean, the latter sample represents the closely related Pacific herring. This study has revealed a large number of genes related to the adaptation to the Baltic Sea as well as to variation in spawning time (spring vs. autumn spawners). The Atlantic herring has turned out to be an outstanding model to study the genetic basis for adaptation due to the huge populations size and the lack of genetic differentiation at selectively neutral loci.

Some of the projects are carried out in collaboration with Drs. Carl-Johan Rubin, Matthew Webster and Kerstin Lindblad-Toh at IMBIM and with several external collaborators.

MOLECULAR COAT COLOUR GENETICS

Susanne Bornelöv, Freyja Imsland, Susanne Kerje, Jessica Pettersson, Doreen Schwochow, Elisabeth Sundström, Leif Andersson

Coat colour variation has been extensively used during the history of genetics to study how genes act and interact in shaping phenotypic variation. This is because the phenotypic read-out is so straightforward making it possible to establish high-resolution genotype-phenotype relationships as well illustrated by our track record in this field. A hallmark of domestic animals is extensive coat colour diversity. We have taken advantage of this and characterized a large number of mutations causing coat colour phenotypes in various domestic animals. At present, we are working with the following phenotypes: (i) Sex-linked barring in chicken, which is controlled by mutations in the *CDKN2A* tumour suppressor gene; (ii) the patterning phenotype in chicken; (iii) inhibition of gold in chicken; (iv) white spotting in dogs controlled by *MITF*, encoding a transcription factor of crucial importance for pigment cell development and function; (v) white spotting in pigs controlled by the *KIT* locus encoding a tyrosinase kinase receptor; (vi) roan coat colour in horses, controlled by a regulatory mutation in the *KIT* gene; (vii) dun coat colour in horses, dun is the wild-type colour in horses and is characterized by dilution of pigmentation, a dorsal black stripe and occasional zebra-like leg stripes; (viii) variant red in cattle. In all these projects our ambition is to nail down the causal mutation(s) and explain the mechanism of action for the detected mutations.

These projects are carried out together with numerous collaborators in Sweden and abroad.

GENETIC ANALYSIS OF THREE CHICKEN MODELS FOR AUTOIMMUNE DISORDERS IN HUMANS

Susanne Bornelöv, Miriam Friedman-Einat, Ulla Gustafson, Susanne Kerje, **Mårten Larsson, Rakan Naboulsi, Jessica Pettersson, Leif Andersson**

We have initiated 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 Dr. Olle Kämpe at Karloinska Institutet and Dr. Örjan Carlborg at SLU.

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

Anna Hjälm Golovko, Elisabeth Sundström, Leif Andersson

Grey is a dominant coat colour mutation that is common in horses and found in a variety of breeds including Arabian horses, Lipizzaner 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 nuclear receptor. We are currently exploring the mechanism leading to premature greying and melanoma development. We have recently established a mouse knock-in mouse where the horse mutation (a 4.5 kb duplication) has been introduced at the orthologous position in the mouse genome. The work is carried out in collaboration with researchers at University of Natural Resources and Applied Life Sciences, Vienna.

GENETIC ANALYSIS OF DIVERGENT INTERCROSSES OF CHICKEN

Ulrika Gunnarsson, Susanne Kerje, Khurram Maqbool, 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 quantitative trait loci (QTL) controlling growth, body composition, fertility, egg weight, behaviour and bone density. 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).

GENETIC AND FUNTIONAL CHARACTERISATION OF DOG DOMESTICATION

Erik Axelsson

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.

Project worker during 2014

Johanna Axling, Genetic and functional characterisation of a duplication with a potential role in dog behaviour.

Publications 2012 to 2014

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EPIGENETICS AND NEW ANTIFUNGAL DRUGS

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. Still, very little is known about how to reverse disease-causing changes in the epigenome. 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. In addition we are investigating the function of the newly discovered transcription factor ZBED6 in mammalian cells in collaboration with Leif Anderssons group.

We have also initiated a project with the long-term goal of developing new drugs against yeast, since severe systemic yeast infections is a growing problem in the health care. Several species of the pathogenic yeast *Candida* normally grow on the skin of humans and only people with a lowered immune response, for example immunosuppressed patient undergoing transplantation or AIDS patients, suffer from *Candida* infections. The drugs against *Candida* frequently give strong side effect and resistance to the drugs is increasing, so new drugs would be of great importance.

Members of the group during 2014
Pernilla Bjerling, group leader
Vladimir Maksimov, post-doc
Alejandro Rodriguez, post-doc
Daniel Steinhauf, PhD student

Projects students during 2014
Frida Forsberg
Robin Jonsson
Dennis Larsson
Marcus Wäneskog
Pik Kei Yuen

Publications 2012 to 2014

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Agencies that support the work
The Swedish Cancer Society
The Swedish Research Council for Medical Research
The Swedish Research Council for Science and Technology
Göran Gustafssons Stiftelse

FORMATION OF REPRESSIVE CHROMATIN

Vladimir Maksimov, Alejandro Rodriquez, Daniel Steinhauf

In *Schizosaccharomyces pombe* there are several regions where a special form of transcriptionally repressed chromatin, named heterochromatin, is formed. The formation of heterochromatin results in transcriptional silencing of reporter genes inserted into that region of the chromosome. 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, primarily SUV39H in humans, and Clr4 in *S. pombe*. The H3K9Me2/3 modification creates a binding site for chromodomain proteins of the HP1 subfamily. Moreover, the SNF2-HDAC repressor complex (SHREC) is crucial for heterochromatin formation in *S. pombe*. SHREC contains a nucleosome remodeller, Mit1, the histone deacetylase Clr3, the HP1 homologue Chp2, the uncharacterised Zn-finger containing protein Clr1 and Clr2. The function of the three last proteins in the SHREC complex remains to be elucidated. We have together with collaborators made strains that carry point mutations of critical, conserved amino acids in these three proteins. The effect on silencing in the strains with point mutations in the Clr2 protein has been characterised (Steinhauf et al 2014). Several of the Clr2 mutant strains display unstable silencing phenotypes indicating deficiencies in either establishment or maintenance of heterochromatin. We are now investigating whether these point mutations in Clr2, as well point mutations in Chp2 or Clr1, affects the integrity of the SHREC complex or the SHREC complex ability to bind to chromatin. We think that these will help to clarify the role of the SHREC complex in the establishment as well as the maintenance of heterochromatin. This will then advance our knowledge how switches between different epigenetic states can occur.

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 the yeast 2-hybrid system and biochemical purifications. The project is carried out in collaboration with Leif Andersson and his group.

HiSTIDINE KINASES IN S. POMBE AND CANDIDA ALBICANS

Pernilla Bjerling, Vladimir Maksimov, Alejandro Rodriguez

Bacteria, plant and yeast have on their cell surface histidine kinases that act as environmental sensors. *S. pombe* has three histidine kinases that are known to be important for the response to nitrogen starvation. Our group has previously investigated the effect on gene regulation and chromatin changes during nitrogen starvation in *S. pombe*, and our findings are published in two papers (Alfredsson-Timmins et al JCS 2007 and Kristell et al GR 2011). Now we are focusing on understanding how these histidine kinases sense the environmental change during nitrogen starvation as well as during other type of changes, for example during osmotic stress. Moreover, in other yeast species like the pathogenic yeast *Candida albicans* the histidine kinases are important for the transition between yeast (unicellular) to hyphal (multicellular) growth. This makes histidine kinases important virulence factors, since this transition need to occur in order for *C. albicans* to penetrate the human skin. *C. albicans* normally grow on the skin of humans and only people with a lowered immuneresponse, like immunosuppressed patient undergoing transplantation or AIDS patients, suffer from *C. albicans* infections. The drugs against *C. albicans* frequently give strong side effect so improved formulas would be of great importance. The histidine kinases are not found in higher eukaryotes and therefore they are promising drug targets. This project aims to set up a drug screen against the histidine kinases in *C. albicans*.

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. Since 2013, the group is host to the BILS Genome Annotation Centre, funded by the Bioinformatics Infrastructure for Life Sciences (BILS). In the fall of 2014, the BILS Genome Assembly Centre was added to the group.

Members of the group during 2014
Manfred G. Grabherr (group leader)
Neda Zamani
Görel Sundström
Marc P. Höppner
Henrik Lantz
Jacques Dainat
Thomas Källman
Martin Norling

Publications 2012 to 2014

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DNA AND RNA SEQUENCE ASSEMBLY

Neda Zamani

Next generation sequencing technologies allow for generating large amounts of RNA or DNA data at low cost. However, the sequenced fragments are short and require assembly into larger, continuous sequences. Powerful existing sequence assemblers are built on the concept of the *de Bruijn* graph, in order to compress the data so that it can be processed in finite amounts of computation time. This method, however, is limited by the graph complexity, which hampers accurate reconstruction of closely related gene paralogs, either stemming from duplications within a single species, or a metagenomics or metatranscriptomics data set from mixed species. We have developed an alternative algorithm and software, *Ananas*, which

efficiently computes possible read-read overlaps dynamically and without the need for hash- or k-mer-tables, which typically consume significant amounts of computer memory. In addition, we eliminated the use of fixed-size entities or sub-sequences, as required by *de Bruijn* graph based methods, resulting in optimal specificity and minimal complexity. Thus, our algorithm is suitable for deployment on third-generation sequencing technology that produce longer reads, but at higher average error rates. The modular architecture of the software allows for assembling both genome sequences as well as transcriptomes, with initial results indicating that we accurately recover complex sequences that were missed by existing assembly programs, both on DNA and RNA data sets.

TYPE I DIABETES GENE EXPRESSION STUDY

Görel Sundström

In this collaboration with Olle Korsgren, Oskar Skog, Sofie Ingvast (Uppsala University), Lars Krogvold (Oslo University Hospital), and others, we examine the differences in gene expression patterns in pancreatic islets between patients with early onset type I diabetes and non-diabetic controls. While insulin production is reduced due to down-regulation of the *INS* gene in the diabetics, we also found two upstream regulators expressed at lower levels, as well as parts of the *MAPK* pathway. Interestingly, genes in the class II major histocompatibility complex genes show significantly higher activity, but not class I or class III genes, indicating a rather narrow and specific immune response, albeit the cause to which the immune system responds remains unclear. In addition, we find differences in a number of other genes linked to inflammatory response. Further analysis on a pathway level promises to shed light on the biological programs that differentiate diabetic pancreatic islets from non-diabetic tissue.

BILS

Thomas Källman

Thomas is working full time for BILS focusing on RNA-sequence analysis, including support, pipeline development and teaching. Projects include RNA-sequencing aiming at validating splice variants from exome arrays in Mouse, two projects looking at gene expression dynamics in zebrafish, and analysis of gene expression during development of *Plasmodium falsiparum*. Other tasks include giving feedback on grant applications as well as helping out in questions regarding experimental design for three RNA sequence projects. On the teaching side Thomas has created a new data set to be used in teaching the course “Introduction to Bioinformatics using NGS data” and acted both as a lecturer and TA as the computer lab for that course at two different occasions. Finally, there are active discussions with developers at BILS to build pipelines for RNA-seq analysis using the scripting language b-pipe.

BILS UPPSALA GENOME ANNOTATION CENTRE

Marc Höppner, Jacques Dainat

The Grabherr group is host to the BILS genome annotation platform, established in August 2013 and managed by Marc Höppner. The primary goal of the platform is to enable and support genome-scale research, specifically genome projects, conducted in Sweden or with Swedish participation. Activities of the platform can be divided into development of hard- and software infrastructure on the one hand and developing a robust workflow for generating computational gene builds and making them available to the collaborators on the other. At the time of writing, the platform has completed – or is in the process of completing – 11 annotation projects, including three vertebrate and 4 plant species. In addition, the platform has served as consultant and collaborator on several additional projects.

BILS UPPSALA GENOME ASSEMBLY CENTRE

Henrik Lantz, Martin Norling

Funded by the Bioinformatics Infrastructure for Life Sciences (BILS), the Uppsala Genome Annotation Centre was started in the summer of 2014 with the mission to provide high quality genome assembly to Swedish genome sequencing projects. This group, managed by Henrik Lantz, both utilizes state-of the art annotation software, as well as exploring new routes to best assemble data from large-scale DNA sequencing efforts.

RETROVIRUS-HOST EVOLUTION

Patric Jern

The overall aim is to better understand the evolutionary interactions among retroviruses and their host species. Retroviruses, such as HIV in humans, must become part of the host cell's genome to produce new viruses. When a germline cell is infected there is a chance for the retrovirus to be passed on to the host's offspring as an inherited endogenous retrovirus (ERV). Consequently, retroviruses have colonized host genomes for millions of years, leaving traces as ERVs in their genetic make-up. These ERVs provide a unique resource for understanding the biology and evolution of virus-host relationships. We mainly employ bioinformatics to study evolutionary retrovirus-host associations along two lines of research:

I. How did our ancestors deal with their pathogens?
We perform comparative studies across the genomes of diverse host species to construct evolutionary hypotheses of relationship and explore retrovirus features, dynamics and transmission for insights into evolutionary retrovirus-host interactions.

II. What are the evolutionary effects of retrovirus integrations on host biology?
We characterize ERVs and other transposable genetic sequences across diverse host genomes in order to elucidate the contributions that they have had on host genomic variation and innovation, and to evaluate their contributions to host biology and phenotypic evolution.

Members of the group during 2014
Patric Jern, associate professor
Alexander Hayward, researcher (until August 2014)

Publications 2012 to 2014

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The Swedish Research Council FORMAS.
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The Medical Faculty, Uppsala University.
Marcus Borgström's Foundation.

RETROVIRUS AND TRANSPOSABLE SEQUENCE EVOLUTION

Alexander Hayward, Patric Jern

The expanding catalogue of re-sequenced genomes and reference assemblies permits detailed comparative studies across the genomes of diverse organisms. We take advantage of this resource to characterize ERVs and other transposable genetic sequences in order to identify novel broad-scale patterns and processes of evolutionary importance. Specifically, we seek to elucidate retroviral spread during evolution and contributions that retroviruses and transposable genetic sequences 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 other transposable sequences, 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 of 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. We have just started sequencing an additional 150 mammals to reach a total of 200 mammals and single base constraint resolution. The majority of the sequencing will be performed in Uppsala. We also study genome evolution across vertebrates to understand how genomes change and allow organisms to adapt to novel environments. This year a lot of emphasis has been on 1) the rabbit genome allowing the study of genes involved in rabbit domestication revealing thousands of non-coding allele frequency differences affecting brain development and function and 2) the cichlid genome projects examining the diverse changes linked to the dramatic phenotypic diversity and species radiations seen in African lakes. This project points to the importance of regulatory variants and standing variation for adaptation to novel environments.

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 aetiology 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 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 evaluating these genetically and functionally. In parallel, we are now studying patient cohorts to identify disease mutations in genes found in dogs.

Members of the group during 2014
Kerstin Lindblad-Toh, Professor, group leader”
Cecilia Johansson, Project coordinator
Eva Murén, Senior Research Engineer
Åsa Karlsson, Research Engineer
Sergey Kozyrev, Researcher, group leader
Maja Arendt, Researcher
Marcin Kierczak, Researcher
Brita Ardesjö-Lundgren, Researcher
Malin Melin, Researcher
Lina Hultin-Rosenberg, Bioinformatician
Johanna Dahlqvist, Postdoc
Fabiana Farias, Postdoc
Ingegerd Elvers, Postdoc”
Emma Ivansson, Postdoc
Nina Oparina, Postdoc
Hanna Bremer, Graduate Student*
Argyri “Iris” Mathioudaki, Graduate Student
Katherine Megquier, Graduate Student”
Katarina Tengvall, Graduate Student
Jessika Nordin, Project Assistant
Sharadha Sakthikumar, Project Assistant
Veronika Scholz, Project Assistant

”dual affiliation with the Broad Institute
*primary affiliation Swedish University of Agricultural Sciences

Project worker during 2014
Madeleine Bergstedt, Sofosko student
Erica Nyberg, research training

International exchange during 2014
Kerstin Lindblad-Toh (Broad Institute)
Ingegerd Elvers (Broad Institute)

Publications 2012 to 2014

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CANCER

Maja Arendt, Ingegerd Elvers, Emma Ivansson, Argyri Mathoiudaki, Katherine Megquier, Malin Melin, Sharadha Sakthikumar

CANINE CANCER

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
- Hemangiosarcoma

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 and fine-mapping 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 mutations and expression level alterations

in the canine tumours. This will provide us with important information on common cancer pathways and therapeutic targets.

At the end of 2014 an article exploring the genetics of hemangiosarcoma and B-cell lymphoma in Golden retrievers was accepted in PLoS Genetics. We identified shared risk factors at two nearby loci, predisposing to both B-cell lymphoma and hemangiosarcoma in Golden retrievers. Resequencing of nine individuals showed no coding mutations in the regions following either any of the risk haplotypes or the phenotype. Differential gene expression studies of tumours show that the risk haplotypes at both loci are associated with an effect on immune system regulation.

BREAST CANCER

From all the types of human cancer, breast cancer (BrCa) is the most common among women worldwide and the second cause of death among female cancer patients. Due to the many similarities of canine mammary tumor (CMT) and BrCa, dogs have emerged as a complementary model to investigate the genetic basis of this cancer in humans and to understand the biology of tumor progression further. Together with Tobias Sjöblom, UU and Annika Lindblom, KI, we are performing a comparative genetic study; extending the benefits from the genetic studies performed in the dog model to humans. We are performing targeted resequencing of the top associated loci from the CMT GWAS, genes in top candidate pathways targeting exons, UTRs and evolutionary conserved regions in the vicinity. The study focuses both on germ-line mutations in Swedish BrCa patients, and on tumor/normal comparison for somatic cancer mutations that may affect carcinogenesis, metastasis and disease prognosis. All in all, our ultimate goal is to identify new genetic risk factors in human disease that could be used either as more effective and reliable screening tests before the disease manifests or as novel diagnostic and/or prognostic targets.

AUTOIMMUNE AND INFLAMMATORY DISEASES

SYSTEMIC LUPUS ERYTHEMATOSUS

Sergey Kozyrev, Fabiana Farias, Hanna Bremer, Johanna Dahlquist, Nina Oparina, Lina Hultin-Rosenberg

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder characterized by dysregulation of the immune system, which results in production of autoantibodies, generation of toxic immune complexes, 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 childbearing age than in men (9:1). While SLE was first described in human patients, it is also observed in other species including dogs with similar clinical manifestations. We have identified eleven genes associated with SLE in Nova Scotia duck tolling retrievers, a dog breed strongly predisposed to an SLE-like disease called immune-mediated rheumatic disease (IMRD). Further, we found that particular sub-phenotypes of IMRD, based on homogenous and speckled immunofluorescent antinuclear antibodies staining pattern, are associated with different but overlapping sets of genes, suggesting not only immunological but also genetic differences associated with various disease manifestations.

In parallel we study genetics of human SLE by targeted resequencing of candidate genes in a large cohort of human SLE patients, where phenotypic sub-classification is available (see abstract below).

Finally, the Kozyrev's group is focused on the analysis of exomes of Islandic patients with severe highly penetrant familial form of SLE. We also identified a novel SLE susceptibility gene *ABHD6* coding for a protein acting in the endocannabinoid signaling pathway, and study the gene functions related to autoimmunity and the role of endocannabinoid pathway in the immune system responses.

SUB-CATEGORIZING INFLAMMATORY DISEASE BY MOLECULAR PATHWAYS

Fabiana Farias, Johanna Dahlquist, Åsa Karlsson, Sergey Kozyrev, Argyri Mathoudaki, Jennifer Meadows, Eva Murén, Jessika Nordin, Gerli Rosengren-Pielberg, Lina Hultin-Rosenberg

We have selected a set of 1900 genes from our canine immunological disease models as well as the genes and pathways implicated by corresponding human disease studies. We are sequencing these genes and the non-coding conserved elements within 100 kb in different human disease cohorts. Our goal is to identify both common and rare disease variants. By looking at carefully phenotyped human patient cohorts and a distinct and comprehensive gene set we expect to start to link diseases and subphenotypes to mutations in specific genes and pathways, possibly allowing a more comprehensive view of the molecular pathogenesis. In a pilot study targeting a subset of the genes, we detected a large number of novel SNPs. Ten SNPs in 6 genes were selected as the best regulatory candidates where the SNP may alter transcription factor binding, only one of those genes has been previously associated with human SLE. Patient history was analyzed in conjunction with SNP and gene information, and in some cases point to a correlation with specific sub-phenotypes. Functional analysis shows differential binding between the reference and mutant alleles for one of our candidate SNPs as well as differential luciferase expression between reference and mutant allele for multiple SNPs. Overall the data suggests a potential function of these SNPs in specific cell types related to certain sub-phenotypes. These candidate SNPs now need to be further studied functionally and be genetically validated in a larger cohort of patient and controls.

ATOPIC DERMATITIS

Katarina Tengvall, Marcin Kierczak, Fabiana Farias, Sergey Kozyrev, Eva Murén, Brita Ardesjö-Lundgren

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 shepherd dogs (GSDs). We also measured the IgA levels in the serum of the same individuals and noted a high correlation between serum IgA and CAD phenotype. The GWAS (using IgA levels and age at sampling as a covariate) generated a genome wide significant association to a locus on CFA27. Fine-mapping of this 1.5 Mb region pinpointed the candidate gene *PKP2* encoding the protein Plakophillin 2, important for maintaining strong skin structure. Currently, we are

collecting skin biopsies from case and control individuals to be used for RNA studies and immunohistochemistry with the specific aims of evaluating the *PKP2* expression in the skin. In addition, we are using EMSA and Luciferase techniques to functionally evaluate two candidate mutations that are predicted to cause an over-expression of *PKP2* in GSDs.

ADDISON'S DISEASE

Katarina Tengvall, Jeanette Hansson (SLU)

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. We have also performed the largest epidemiological study of canine adrenocortical insufficiency (AI) based on ~500,000 Swedish dogs insured by AGRIA. In this study we present data supporting the presence of breed-specific differences in AI regarding incidence rates, gender distribution and survival fractions, indicating the existence of different subtypes of AI in the dog, in analogy to what is known in people.

METABOLIC AND CARDIOVASCULAR DISEASE

DIABETES

Maja Arendt

Hormone induced diabetes can develop in female dogs post estrous or during 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 (GWAS) 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. We have also performed whole genome sequencing in a subset of Swedish Elkhound cases (18) and controls (12) and identified many variants in potential candidate genes. We are currently comparing the GWAS associated regions with the whole genome sequencing in order to identify causal variants, which are associated with the disease. Long term we are aiming to identify disease-associated genes leading to better understanding of diabetes in general, as well as improved treatment opportunities and actions for disease prevention, which could benefit both human as well as dogs.

DILATED CARDIOMYOPATHY

Jennifer Meadows, Suzana Steila (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.

GWAS results from 182 GDs and 133 NFs has allowed for the identification of associated regions. Targeted genome sequencing was performed and the role structural variants play in disease is currently being assessed. Genes of interest from the canine cardiac project are being carried forward into a human targeted sequencing program where the collection of Swedish cases and controls relative for Sudden Cardiac Death is ongoing.

NEUROLOGICAL AND BEHAVIOURAL TRAITS

DEGENERATIVE MYELOPATHY

Emma Ivansson, Katherine Megquier, Sergey Kozyrev, Eva Murén

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 (PWC) breed and further validation in other breeds. A mutation in human *SOD1* causes a similar neurodegenerative disease, amyotrophic lateral sclerosis (ALS), suggesting 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. We have performed additional GWAs in carriers of the SOD1 risk allele in the PWC breed. We identified a novel modifier gene, which affects both risk of disease and age of onset. Several newly identified variants within this gene have regulatory potential, altering transcription factor binding and/or overall isoform balance and gene expression. We are collaborating with Ingela Nygren at Akademiska Hospital and Peter Andersen at Umeå University to follow up genes and pathways identified in the canine breeds in human ALS patients.

INVESTIGATING GENETICS UNDERLYING BEHAVIORAL TRAITS IN DOGS

Marcin Kierczak, Katarina Tengvall, Fabiana Farias

Domestic dog has been accompanying man for more than two thousand years. Two events in this common history are particularly important: the domestication and the creation of modern breeds. Both events involved selection of certain, often behavior-related traits and features.

Since 1989 the Swedish Working Dog Association has been carrying Dog Mentality Assessment (MH) test, in which different aspects of behavior are measured, e.g. intensity of social contact, playfulness or eagerness to chase.

Encouraged by the results of our pilot studies involving 200 German shepherds (GSDs), we worked together with dog owners to collect and genotype more individuals to enable studies of even subtle polygenic effects. In our latest study, we collected data from nearly 500 shepherds and we found out that behavior-related traits are often controlled in an oligogenic or polygenic manner. The associated genes are often involved in the same genetic network or biological process. Perhaps the most exciting findings so far point us to the genes involved in the early development of the nervous system as well as to genes involved in signal transmission within the nervous system and at the junction of the nervous system end effector cells.

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

GENETIC DISSECTION OF AUTOINFLAMMATORY DISEASE

Jennifer Meadows

The long-term research goal is to identify the genes and molecular variants that underpin traits of importance to both the health and well being of companion animals. Paired with this is a comparative genetics aspect, where through international collaborative efforts, research findings are translated to aid human patients with orthologous disease sets.

Our current projects leverage knowledge gained from studying the purebred form of the domestic dog. The dog represents an excellent model organism for human comparative analyses since they share roughly the same gene set, develop spontaneous disease with similar aetiologies and generally share the same environment. The true benefit to using purebred dogs lies in the exploitation of the genetic structure honed through selection bottlenecks, where long within breed haplotypes and the enrichment of genetic risk factors mean that the trait mapping of heterogeneous diseases can be investigated with both fewer markers and fewer individuals than is possible for human genetics.

The two main projects investigate the molecular genetics of canine and human forms of autoinflammatory disease (AID). These diseases are characterised as unprovoked episodes of inflammation associated with abnormal regulation of innate immunity (i.e. absence of high-titre autoantibodies or antigen-specific T cells).

Members of the group during 2014
Jennifer Meadows, Researcher, group leader
Argyri "Iris" Mathioudaki, PhD student

Project worker during 2014
Jessika Nordin, Project worker
Daniela Hahn, Project student

Publications 2012 to 2014

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SHAR-PEI AUTOINFLAMMATORY DISEASE (SPAID)

Jennifer Meadows

Autoinflammatory disease results from the dysregulation of the innate immune system: the body's first line of defiance against infection. The clinical picture of SPAID in purebred Shar-Pei dog is typically 6-72 hours-long attacks of high fever and other signs of inflammation (especially around the hocks). During these attacks the animal shows reluctance to move and is generally indolent. Within 24 hours, the dog is again alert, remaining asymptomatic between episodes. Shar-Pei with SPAID have consistently elevated levels of the cytokine IL-6 and can be treated with blockers of IL-1B, such as colchicine.

Persistent inflammation in affected Shar-Pei can lead to reactive amyloidosis, the accumulation of aberrantly produced acute phase proteins in multiple organs. These aggregates are particularly damaging to the kidney and can result in organ damage and ultimately organ failure. The clinical picture is varied. Some individuals may be susceptible to amyloidosis without having other symptoms of SPAID and vice versa.

Our understanding of the genetics that underpin Shar-Pei health has rapidly evolved over the past five years. Since our 2011 publication, which reported that a shared locus was linked to both Familial Shar-Pei Fever (FSF) and the dogs' classical thickened and wrinkled skin, we now understand that Fever is only one of the signs of Shar-Pei Autoinflammatory Disease (SPAID). We have used discrete genome wide association analyses to show that a single genetic locus predisposes the breed to many types of persistent inflammation, in addition to recurrent fevers, including arthritis, Shar-Pei specific secondary dermatitis (hyaluronan filled vesicles affecting the skin, termed vesicular hyaluronosis), otitis and systemic reactive amyloidosis. Our most recent research has been geared towards using whole genome sequencing to resolve the difference between breed subtypes and disease associated alleles. We have also been refining a genetic test for SPAID and investigating the potential genetic signatures of early and late onset renal amyloidosis.

PATHWAYS OF HUMAN INFLAMMATORY DISEASE

Jennifer Meadows, Iris Mathoudaki, Jessika Nordin

Ankylosing Spondylitis (SpA) is one of a growing number of human polygenic autoinflammatory diseases. It is manifested by chronic spinal and sacroiliac joint arthritis, which in time will result in the loss of mobility due to spinal fusion and potentially, restrictive lung disease. Inflammation may also affect peripheral joints and nonarticular structures, presenting clinically as enthesitis or uveitis. SpA is highly heritable (>90%) and with an affliction rate of 1/200 within Europe, is one of the most common forms of inflammatory arthritis. The genetics of disease have long implicated HLA-B27, however 10% of the European population carries this subtype, but only 1-5% of those develop SpA.

In 2014, we completed the sequencing of ~400 carefully phenotyped SpA patients and a similar number of matched controls using the 1900 gene ImmunoArray. This "array" is a targeted liquid capture library which, with a combination of coding and regulatory regions, covers ~32Mb of the human genome. Illumina paired end sequencing was used to sequence each individuals to an average depth of 30x depth. A robust bioinformatic pipeline moving from fastq raw reads to vcf formatted annotated variants has been designed and implemented at UPPMAX. We now stand ready to evaluate our identified variants in the context of those common globally (1000 genomes/dbSNP) and those common to our patients' specific region of Sweden (using our control set). The rare disease associated variants identified by this project may prove useful in both the diagnostic and treatment fields.

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 2014
Gerli Rosengren Pielberg, Assistant Professor, group leader
Matteo Bianchi, PhD student

Project students during 2014
Hannes Hällgren
Helena Zetterqvist

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CHARACTERIZATION OF GENETIC RISK FACTORS BEHIND CANINE 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 potentially contributing to development of human Thyroiditis. We have performed a genome-wide association analysis and identified several candidate loci in different dog breeds. Currently we are in the process of identifying potential candidate mutations by next generation sequencing of key individuals representing the risk/protective haplotypes. Identified candidate mutations will be screened in larger sample cohorts and functionally evaluated.

The results from this study may lead to development of genetic tests and better diagnostic methods as well as new alternative therapies for treatment and breeding guidelines of dogs.

IDENTIFICATION AND CHARACTERIZATION OF GENETIC RISK FACTORS BEHIND HUMAN AUTOIMMUNE POLYENDOCRINE SYNDROMES

Matteo Bianchi, Gerli Rosengren Pielberg

Human autoimmune polyendocrine syndromes are a heterogeneous group of diseases characterized by autoimmune activity against more than one endocrine organ. In collaboration with Prof. Olle Kämpe (Karolinska Institutet), we have started a project for characterizing genetic risk factors contributing to the development of these syndromes.

We are taking advantage of knowledge gained from comparative genetics (more specifically canine genetic risk factors and involved pathways). By using modern high-throughput techniques, such as hybrid capture and next-generation sequencing, we will perform a thorough screening of mutations in about 1900 candidate immune genes and their regulatory elements in Swedish Addison patient cohort of more than 700 individuals.

The results from this study will provide us knowledge about the sharing of genetic risk factors behind immunological diseases in different species, as well as potentially identify new genes and pathways important in development of such diseases in humans.

FUNCTIONAL GENOMICS

IDENTIFICATION AND CHARACTERIZATION OF GENES AND MECHANISMS CONTROLLING PHENOTYPIC TRAITS

Carl-Johan Rubin

The overall aims are to explain how genetic variation impacts diseases and phenotypic traits and to explore molecular processes affecting how the genetic code is processed depending on environmental factors. In one project, massively parallel DNA sequencing is utilized in order to identify genes underlying phenotypic variation and disease in the horse. The most important findings made during the course of this project will be further pursued and validated in collaboration with breeders and/or veterinarians. In another project we investigate the contribution of genetic and environmental factors on phenotypic variation in Atlantic salmon using genetic-, epigenetic- and gene expression profiling. Phenotypes assessed include time of sexual maturation, growth, and various behavioral traits. For this project we will include clonal lines as well as wild salmon in order to study the impact of environmental difference on traits with and without confounding effects added by genetic variation. Furthermore, the integration of genetic, epigenetic and transcriptome profiling gives a first opportunity of deep exploration of Atlantic salmon genome biology; including prevailing gene regulatory and epigenetic mechanisms.

Members of the group during 2014
Carl-Johan Rubin
Markus Sällman Almén

Agencies that support the work
The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning
The Research Council of Norway

GENETICS OF DISEASE, MORPHOLOGY AND PIGMENTATION IN HORSES

Carl-Johan Rubin

Millennia of human-imposed selective breeding for desired traits has altered the phenotypic repertoire of the horse for traits such as size, body conformation, behavior, and color, with such variation being conferred by changes in frequencies of alleles at mostly unknown genetic loci. Lately, new methods for DNA sequencing have emerged and it is now possible to determine near-complete sequences of large numbers of mammalian genomes in parallel.

The major aims are to generate a fine-scale map of genetic variation in the horse (*Equus Caballus*) genome, and to analyze patterns of genetic variation in horse breeds in order to detect loci affected by selection and to detect genetic variants contributing to specific traits and diseases. To achieve these aims we have sequenced DNA samples from diverse horse breeds and populations, selected to represent distinct disease/trait classes. Samples were subjected to whole genome resequencing (WGS) and obtained sequences were used in genome scans to detect signatures of selection and functional polymorphisms/mutations. We predict functional genetic variants using bioinformatics methods and screen for alleles uniquely/preferentially observed in individuals expressing certain diseases or traits. For such

candidates we proceed with association analysis in larger cohorts of horses to investigate whether identified candidate alleles are significantly associated with the traits.

The project is carried out in collaboration with Sofia Mikko at the Swedish University of Agricultural Sciences

EPIGENETICS AND OTHER MEANS OF ALTERING GENOME UTILIZATION IN RESPONSE TO ENVIRONMENTAL VARIATION

Carl-Johan Rubin, Markus Sällman Almén

GENETIC AND EPIGENETIC CONTRIBUTION TO MALE SEXUAL MATURATION AND BEHAVIOR

Farmed Atlantic salmon (*Salmo salar*) has been the subject of intense selection for increased growth, but selection alone has not sufficed to counteract frequent occurrence of reduced growth in response to early puberty. Reduced growth due to premature maturation has been mitigated by specific light regimens. It is assumed that maturation is also modulated by variation in water temperature, which could be detrimental to commercial Salmon breeding as sea temperature is expected to rise due to global warming. One mechanism by which environmental factors can influence the expression of phenotypic traits is through epigenetic modifications of DNA in animal cells, thereby affecting the activity of genes.

In this project we use massively parallel sequencing (genome sequencing, whole genome bisulfite sequencing, Reduced Representation Bisulfite Sequencing (RRBS), RNA-sequencing and micro-RNA sequencing) in order to, in clonal individuals, investigate changes in epigenetic marks and gene expression signatures accompanying exposure to environmental variation, including different light- and temperature regimens and stress tests during different life stages in the Atlantic salmon. Furthermore, wild salmon are known to differ for their time of sexual maturation and the genetics of this difference is assessed in screens using whole genome resequencing of pools comprising early- vs. late sexually maturing individuals from rivers across Norway. The combination of global genetic-, epigenetic- and transcription profiling provides an integrated view of Atlantic salmon genome biology and makes it possible not only to study factors driving behavior and maturation, but also to provide a first functional context in terms of gene expression and regulation.

In order to evaluate the contribution of genetic- as well as epigenetic factors on a trait as complex as behavior we are utilizing lines exhibiting behavioral differences as well as clonal fish, since the latter makes it easier to study epigenetic effects without genetic variation acting as a confounder. To obtain relevant phenotypic read-outs we are utilizing PIT-tags, i.e. sensors capable of determining spatial location of individual fish in tanks or sea-cages. Data from these sensors can be used to approximate traits such as activity, feeding, swim depth etc. and these traits will be correlated with life history events as well as with read-outs from genetic- and epigenetic screens, i.e. alleles or epi-alleles.

These projects are conducted in collaboration with researchers at the Institute of Marine Research in Bergen, Norway.

GENOME EVOLUTION

Matthew T Webster

We study evolution on the molecular level by analysing patterns of genetic variation on the whole-genome scale, using bioinformatic and statistical approaches. We are interested in identifying genetic variants that have been affected by natural selection and analysing the phenotypes they produce. We are also interested in understanding the mechanisms and evolutionary consequences of genomic variation in meiotic recombination.

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 and genetic variants important for adaptation to climate and disease, which could be vital to protect this important species from colony losses. We are using transgenic fruit flies to analyse the effects of these variants. We are also investigating the causes and consequences of extremely high recombination rates in honeybees. Finally, we have recently shown that patterns of DNA methylation are important in controlling recombination events in the dog genome.

Members of the group during 2014
Matthew Webster, group leader
Andreas Wallberg, postdoc
Martin Schmid, postdoc
Jonas Berglund, PhD student

International exchange during 2014
Takashi Makino, visiting researcher (Tohoku University, Japan)
Olaf Thalmann, visiting researcher (University of Turku, Finland)
Dora Henriques, visiting PhD student (Polytechnic Institute of Bragança, Portugal)

Project worker during 2014
Anna Olsson

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

Andreas Wallberg

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.

FUNCTIONAL CHARACTERISATION OF GENETIC POLYMORPHISMS IN THE HONEYBEE

Martin Schmid

We are using the fruit fly *Drosophila melanogaster* as a model to investigate the functional effects of specific genetic variants of evolutionary importance that we identify using selection scans in the honeybee. This is possible to due the vast array of transgenic techniques and mutant lines that are available in *Drosophila* and due to the levels of homology between this species and other insects such as honeybees. We aim to introduce honeybee gene variants into knockout flies and characterise phenotypes using a variety of assays. We are currently focussing on genes involved in metabolism and morphology.

RECOMBINATION AND GENOME EVOLUTION

Jonas Berglund, Andreas Wallberg

Meiotic recombination is a fundamental biological process, which maintains genetic variation within populations and is essential for chromosomal segregation. In many taxa, the genomic distribution of recombination events is localized to specialized sites known as hotspots, but the mechanisms controlling this variability are unclear.

The honeybee has the highest levels of meiotic recombination measured in a sexual eukaryote. The reasons for this are unknown, but it is likely related to an aspect of living in a colony. Recombination may also have damaging effects on the genome, either because it causes structural mutations or due to a process known as biased gene conversion, which alters the frequency of mutations in a population. We are analysing genomic variation in recombination rates in honeybees and other insects in order to understand the evolutionary forces responsible for increasing recombination rates and their effects on genome evolution. We also aim to understand how recombination events are controlled in invertebrates.

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. Our results pinpoint a role of DNA methylation in controlling recombination rate variation.

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, zebrafish and C. elegans.

METHODS FOR MAINTANENCE AND GENETIC MANIPULATION OF PLURIPOTENT STEM CELLS

Cecilia Annerén

Pluripotent stem (PS) cells e.g. embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, offer novel cell sources for basic research, drug toxicity studies, *in vitro* modeling of genetic disorders or therapeutic cell replacement. However, realization of the full potential of stem cells is currently hampered by the difficulty in genetically manipulating as well as routinely culturing these cells. The overall aim of our research is to improve long-term propagation, genetic manipulation and large-scale expansion of PS cells. More specifically, we are: 1) identifying best practices by benchmarking novel and commercially available cell culture media, matrices/surfaces and transfection reagents, 2) delineating the mechanisms involved in stem cell self-renewal and cell attachment by characterizing a newly identified serum protein and identifying its role in these processes and, 3) applying our knowledge to long-term culture using chemically defined reagents. Translation of our findings into commercially available products or methods may potentially be realized via close collaboration with GE Healthcare BioSciences AB (GEHC).

Members of the group during 2014
Cecilia Annerén, Ph.D., Adjunct Senior Lecturer
Sara Pijuan Galitó, Ph.D. student
Christoffer Tamm, Post doc
Sandeep Kadekar, Post doc

Publications 2012 to 2014

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Patents 2014

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Agencies that support our work
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STUDY OF DIFFERENT PROTOCOLS FOR MOUSE EMBRYONIC STEM CELL CULTURE AND TRANSFECTION

Christoffer Tamm, Sandeep Kadekar, Sara Pijuan Galitó

Most stem cell laboratories still rely on early culture methods to support expansion and maintenance of mouse PS 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 and/or use of undefined components. Culture of stem cells under undefined conditions can induce spontaneous differentiation and reduce reproducibility of experiments. In a recent study (Tamm et al, 2013), we compared standard PS cell culture protocols with two newly described ones: 1) growing cells in semi-adherence in a medium containing two small molecule inhibitors (CHIR99021, PD0325901) herein called 2i medium and; 2) growing ES cells in a spheroid suspension culture in a defined medium containing LIF and bFGF herein called ESN2 medium. Our data confirms previous reports showing that the 2i medium generates purer stem cell cultures with negligible signs of spontaneous differentiation, as compared to traditional, FBS-based, mouse PS media. A drawback that we observed with the 2i medium, is that the mouse ES cells are much harder to transfect with standard reagents such as Lipofectamine™ 2000, compared to cells grown in standard serum-containing medium. We are therefore currently evaluating different transfection reagents for plasmid and siRNA on mouse ES cells grown in 2i medium.

ROLE OF INTER-α-INHIBITOR 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 a novel kinase pathway activated by LIF is involved in the maintenance of self-renewal and pluripotency of mES cells (Annerén *et al,*. 2004, Tamm *et al,* 2011 and Tamm *et al,*. 2012). Briefly, we have shown that LIF activates the Src kinase family member Yes, which in turn activates the Yes Associated Protein (YAP). YAP then enters the nucleus and forms an active transcription complex with TEAD2, inducing transcription of other well-described self-renewal and pluripotency factors such as Oct3/4 and Nanog. During our experiments we also found that fetal bovine serum (FBS) can activate Yes and induce TEAD2-dependent transcription in a dose- and time-dependent manner. Through a set of serum fractionations techniques we identified and isolated Inter-α-Inhibitor (IαI). IαI activates Yes/YAP/TEAD pathway by inducing Yes auto-phosphorylation, YAP nuclear localization and TEAD-dependent transcription. The cleaved heavy chain 2 (HC2) sub-component of IαI, was demonstrated to be responsible for this effect. We have also found that addition of IαI or HC2 to the culture promotes mouse and human PS cell attachment under serum-free media conditions, and that PS cells seeded in the presence of IαI can successfully be cultured on uncoated, standard tissue-culture treated plastic. Moreover, IαI successfully supports single cell passaging of human PS cells even without the aid of a ROCK inhibitor molecule. Therefore, we are currently developing a new cell culture protocol for human PS

cells. Until now, 7 different PS cell lines have been adapted to the new media formulation containing IαI, and 4 different human PS cell lines have been grown for over 20 passages and tested for maintenance of pluripotency and genetic integrity. Therefore, we conclude that the new formulation is robust and can maintain and support PS cell culture *in vitro*.

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, zebrafish and *C. elegans* where we 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 2014
Anders Dagälv, postdoc
Tabea Dierker, postdoc
Inger Eriksson, research engineer
Beata Filipek-Górniok, graduate student
Lena Kjellén, professor, group leader
Anders Lundequist, postdoc
Catherine Merry, guest researcher

International exchange during 2014
Emma Lowe, University of Manchester
Beata Filipek-Górniok visited National Human Genome Research Institute, NIH, Bethesda, U.S.A.

Project workers during 2014
Parisa Missaghian (SOFOSKO)
Tong Ha, master student

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REGULATION OF HEPARAN SULFATE BIOSYNTHESIS/ IN SEARCH FOR THE GAGOSOME

Catherine Merry, Tabea Dierker, Parisa Missaghian, 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 features of their heparan sulfate chains. Mice with targeted mutations in the core proteins identified will be used to study functional aspects of the cell surface proteoglycans.

SYNTHESIS AND TRANSPORT IN ZEBRAFISH OF THE SULFATE DONOR PAPS

Beata Filipek-Górniok

PAPS, 3'-phosphoadenosine-5'-phosphosulfate, is the general sulfate donor, needed in all sulfation reactions. It is synthesized from ATP and sulfate by PAPS synthases, located in the cytoplasm and, as recently shown, also in the nucleus. Specific PAPS transporters carry the sulfate donor into the Golgi compartment where sulfation of glycosaminoglycans as well as proteins take place. We have identified three PAPS synthases and two PAPS transporters in the fish and studied their expression. Using morpholino knockdown of gene expression we

identified an important role for one of the synthases in muscle development. This phenotype is further investigated in collaboration with Jonas von Hofsten in Umeå.

TALENs (transcription activator-like effector nucleases) and CRISPR-Cas9 technologies (clustered regularly interspaced short palindromic repeats) are powerful methods allowing site targeted mutagenesis. These novel reverse genetic tools have been used in collaboration with Johan Ledin (Zebrafish platform, SciLife) and Shawn Burgees group (NIH, US) to create zebrafish mutants for two of the PAPS synthases as well as for several heparan sulfate sulfotransferases, giving us the opportunity to study the role of sulfation in this vertebrate model organism.

SULFATE METABOLISM IN CANCER

Anders Lundequist, 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

In collaboration with Andrea Hinas, Department of Cell and Molecular Biology, we use the nematode *C. elegans* to study potential functional overlap between heparan sulfate and chondroitin sulfate. Mutants with defective heparan sulfate biosynthesis show a strong misrouting of motor axons. By introducing chondroitin sulfate sulfotransferases into the mutants we investigate if the routing can be corrected. The nematode synthesizes large amounts of non-sulfated chondroitin, which was the reason for us to select it as a model. However, we have also recently shown the presence of chondrotin sulfate, which has been missed by previous investigators. We are now identifying the responsible sulfotransferases and the core proteins to which the chondroitin sulfate chains are attached.

‘SMART’ BIOMATERIALS FUNCTIONALISED WITH HEPARAN SULFATE

Catherine Merry, Tong Ha, Anders Dagälv

The ability of heparan sulfate to regulate the activity of multiple growth factors and cytokines is a feature that can be exploited in the design of biomaterials for *in vitro* and *in vivo* applications including tissue engineering and regenerative medicine. We have previously developed a method to enable the display of bio-active heparan sulfate on electrospun scaffolds and more recently optimized a simple peptide hydrogel which can be modified to display heparan sulphate to encapsulated cells. I am working with the Kjellén group to investigate the ability of heparan sulfate-functionalised hydrogels to direct stem cell behavior,

combining embryonic stem cells deficient in heparan sulfate biosynthetic machinery developed in Manchester with a library of similar cells isolated by the Kjellén group. These cells help to pinpoint which signaling factors are activated by specific heparan sulfate oligosaccharides and will be used to generate a library of saccharides that can be combined to direct and control stem cell differentiation.

FUNCTIONAL STUDIES OF BLOOD VESSEL GUIDANCE

Johan Kreuger

The overall objective of our research is to increase our understanding of how instructive concentration gradients of signaling proteins control blood vessel formation. In this context, we also study the contribution of inflammatory cells to the angiogenic process. 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 cell migration events central to 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, whole-genome sequencing and biochemical approaches to study directional angiogenesis. We 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 during 2014
Johan Kreuger, group leader, researcher
Johan Heldin, PhD student
Paul O'Callaghan, postdoc
François Binet, postdoc
Rodrigo Hernández Vera, postdoc

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Patents 2012 to 2014
2013 Microfluidic capsule - SE 1350861-9
Describes a fluidic device to determine responses of cells to test substances for diagnostic purposes.

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The Swedish Cancer Society
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The Medical Faculty at Uppsala University

VASCULAR DEVELOPMENT IN RESPONSE TO GROWTH FACTOR GRADIENTS

Paul O'Callaghan, Johan Heldin, Yvette Zarb, Anna Nieto Esteve, Francois Binet, Rodrigo Hernández Vera

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

PROTEOGLYCANS REGULATING TISSUE DEVELOPMENT

Paul O'Callaghan, Francois Binet, Rodrigo Hernández Vera

Proteoglycans are critical for vascular development as they modulate and potentiate VEGF-receptor mediated angiogenesis. We focus our study on the mechanisms of proteoglycan secretion and the roles of proteoglycans in tyrosine kinase receptor signaling. We have also identified several genes and miRNAs that are selectively expressed either in actively sprouting blood vessels, mural cells, or inflammatory cells. We are now knocking down these candidate genes and miRNAs to identify their roles in the formation, patterning and pathfinding of growing blood vessels.

IN SEARCH FOR GENETIC CAUSES OF PARKES WEBER SYNDROME- A SEVERE VASCULAR ANOMALY

Pär Gerwins, Johan Kreuger, Paul O'Callaghan, 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 their parents have been diagnosed Parkes Weber has been performed, and the search for the disease-causing mutations is currently ongoing. 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 FOR DEVELOPMENT AND DISEASES

Jin-ping Li

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

Members of the group during 2014
Tahira Batool, graduate student (from August)
Hao Cui, PhD, post-doc
Andreas Digre, graduate student
Jianping Fang, PhD, post-doc
Jin-ping Li, MD, PhD, group leader
Ulf Lindahl, PhD, professor emeritus
Tianyi Song, graduate student (from August)

Project worker during 2014
Shilpashree Mallesh, trainee (October - December)

International exchange during 2014
Group member to visit other lab
Jin-ping Li, visited Oncology Department of Beijing Hospital of traditional Chinese Medicine, one week in June, one week in October

Publications 2012 to 2014
1. Li, JP. and Spillmann, D. (2012) Heparan sulfate proteoglycans as multifunctional cell regulators: cell surface receptors *Methods Mol Biol* 836: 239-255
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HEPARAN SULFATE IN ANIMAL DEVELOPMENT

Jianping Fang, Hao Cui

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 HS polymer level. The IdoA units are indispensible for HS binding to ligands, due to the marked conformational flexibility of these residues. Therefore, the reaction catalyzed by 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, HGF and BMPs 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 examining the mechanisms underlying the defects in kidney, lung and skeletal systems.

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

Jianping Fang, Tianyi Song

Biosynthesis of HS is a complex process; concerted action of at least 11 different enzymes results in HS polysaccharide chains with a high degree of heterogeneity. Though same enzymes are expressed in all cells, the structure of HS is highly tissue/cell specific. So, the 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 enzyme complex in the Golgi, denoted as "gagosome".

In this project, particular attention is given to the interactions between enzymes, e.g. GlcA C5-epimerase and O-sulfotransferases by three approaches. 1) *In vitro* studies: recombinant enzymes (GlcA C5-epimerase, HexA 2-O-sulfotransferase and GlcN 6-O-sulfotransferase) are applied to modify polysaccharide substrates for investigation of substrate specificity of the individual enzymes, interaction/regulation of the enzymes in their separate or concerted action towards various substrates and kinetics of the enzymatic reactions. 2) Using authentic cells isolated from transgenic mice defect in the enzymes: 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. 3) Overexpression of the enzymes in cell models: The genes coding for GlcA C5-epimerase, 2-O-sulfotransferase and 6-O-sulfotransferases will be introduced into a cell model, individually or in combined form. The impact of overexpression of the enzymes on HS structure and function will be investigated.

STRUCTURE AND FUNCTIONS OF HEPARAN SULFATE IN AMYLOIDOSIS

Hao Cui, Andreas Digre

"Amyloidosis" refers to a clinical condition encompassing a group of more than 20 post-secretory protein-misfolding diseases. In these diseases, 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 in different patients, it is of importance to find out the functions of HS in these diseases. We primarily focus on inflammation associated amyloid A (SAA) deposition in the spleen/liver/kidney; type II diabetes (IAPP deposition in the pancreas) and Alzheimer's disease (Aβ 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?

Hao Cui, Tianyi Song

Heparanase is an endo-glucuronidase that cleaves HS and heparin. 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 detected in all tissues. At normal conditions, the enzyme is expressed at a relatively low level in most of organs, essentially non-detectable by Western blotting technique, with the exception of platelet and placenta. However, the enzyme is significantly upregulated at several pathological conditions, such as inflammation and cancers. This project aims to find out the functional roles of heparanase with regarding to thrombosis and hemostasis.

Several *in vivo* thrombosis models will be used in our unique 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.

IMPLICATIONS OF HEPRANASE IN RHEUMATOID ATHRITIS

Andreas Digre

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by aggressive proliferation of synovial tissue (ST), which leads 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. Recent study revealed that heparanase level is 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 our unique transgenic mice that are either overexpressing or lacking heparanase for collagen II-induced RA mouse model.

HEPARN SULFATE AND HEPARANASE IN CELL PROLIFERATION AND TUMOR METASTASIS

Tahira Batool, Jianping Fang

Expression of heparanase is upregulated in most human tumor tissues, correlating with increased metastatic potential, tumor vascularity and poor postoperative survival of cancer patients. A direct role of heparanase in tumor progression was demonstrated by increased tumor angiogenesis and metastasis following overexpression of heparanase in the cells, and by marked decrease in the pro-metastatic and pro-angiogenic potentials of cells subjected to heparanase gene silencing. These findings indicate that heparanase is causally involved in cancer progression.

Our earlier studies revealed that the HS chains isolated from tissues overexpressing heparanase had higher activity to assemble FGF2-FGFR complex, suggesting that heparanase promotes functions of the mitogenic growth factors. In this project, we will continue the studies by examination of heparanase effect on cell activities. First, embryonic fibroblast cells (MEF), isolated from the heparanase transgenic mice, are characterized for their proliferation and migration. The signaling activities of growth factors, e.g. FGF2, VEGF and TGF, on the MEF will be analyzed, in comparison with corresponding wildtype MEF cells. Then, HS structures expressed in the MEF cells and other tumor cells that express high levels of heparanase will be characterized.

HEPARANASE AND HS IN FAT METABOLISM AND ATHEROSCLEROSIS

Tianyi Song, Jianping Fang

Atherosclerotic cardiovascular disease is one of major cardiovascular diseases affecting more and more people globally. The disease is pathologically characterized by formation of atherosclerotic lesions that are typically asymmetric focal thickenings of the vessel wall intima. This pathological process involves a complex interplay between lipid metabolism, vessel injuries and inflammation, resulting in the lesions consisting of inflammatory and immune cells, lipids, endothelial and smooth muscle cells as well as proteoglycan complex, e.g. HSPG. Contradictory suggestions have been reported for the roles of HSPG in this pathophysiological process; some studies found HSPG being anti-atherogenic through inhibition of monocytes adhesion and

smooth muscle cell (SMC) growth; while others reported HSPGs having pro-atherogenic effects in mouse models. Thus, more studies are needed for clarification of the pathophysiological functions of HSPG in atherosclerosis.

Our earlier study found increased expression of heparanase in symptomatic carotid atherosclerosis. It is known that acute coronary syndrome and stroke are associated with 'vulnerable plaques' that tend to rupture under certain circumstances. A key aspect of stabilizing these plaques is to have an intact and stable ECM structure that is mainly composed of collagens and HSPG. Our hypothesis is that heparanase degrades HS in the ECM structure of plaques can lead to formation of vulnerable plaques. To approve this, we will feed mice with high fat diet and to investigate lipid metabolism.

THE INVOLVEMENT OF PROTEOGLYCANS AND GLYCOSAMINOGLYCANS IN CANCER AND ANGIOGENESIS

Maria Ringvall

The establishment of a tumor and further progression into a malignant cancerous lesion is dependent on several processes such as dysregulated proliferation, inflammation and angiogenesis. An array of proteins whereof some are proteoglycans are involved in these processes. Proteoglycans are a group of molecules with glycosaminoglycan (GAG) sugar chains attached to a core protein and alterations in expression, structure and glycanation status have been seen in relation to cancer. With the exception of hyaluronan, all GAGs are sulfated and the degree and pattern of sulfation can regulate the capacity to bind to other proteins. Proteoglycans are found in the extracellular matrix, cell surface or intracellularly and are expressed by virtually all cel types. Heparan sulfate (HS) is the most abundant GAG and binds proteins such as growth factors and cytokines in the exracellular matrix and is known to be involved in regulation of cell surface ligand-receptor interactions and activation. Chondroitin sulfate (CS) is another abundant GAG, also with the capacity to bind many different proteins, although the role of these interactions are less well studied than for HS.

Members of the group during 2014
Maria Ringvall, PhD, associate professor
Andrew Hamilton, PhD, postdoc
Vladimir Basic, PhD, postdoc
Kjersti Marie Hjelle, International master student
Alice Haux, second year SOFOSKO student
Alva Sandström, first year SOFOSKO student

Publications 2012 to 2014
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EFFECTS OF HEPARAN SULFATE MIMETICS ON ANGIOGENESIS

Andrew Hamilton, Maria Ringvall

Angiogenesis, the formation of new blood vessels, is an important element during embryo development and wound healing but is additionally a highly active process in different pathological states such as cancer and rheumatoid arthritis. Several signaling systems involved in angiogenesis, such as fibroblast growth factor, vascular endothelial growth factor and platelet derived growth factor can be regulated by interactions between HS and the ligand, or formation of a ternary complex consisting of the ligand and its receptor. The binding properties of HS to other proteins are dependent on the sulfation status of the polysaccharide chain where both total amount of sulfation and sulfation pattern along the glycosaminoglycan chains are of importance. The potential for heparan sulfate to regulate different systems that act during pathological conditions has put forward small, synthetic heparan sulfate mimetics as interesting for drug design. We are now using a group of HS mimetics to evaluate their effect on regulation of the angiogenic process. The design of these mimetics is based on exact positioning of defined sulfated domains separated by a spacing linker molecule. Preliminary results show that such mimetics can have an effect on physiological angiogenesis and we are now continuing to study this aspect together with their effects on pathological angiogenesis.

THE ROLE OF SERGLYCIN IN CANCER

Andrew Hamilton, Vladimir Basic, Maria Ringvall

Serglycin, predominantly a CS-proteoglycan, is the only proteoglycan with a manifested intracellular function where it is known to aid in storage of compounds such as proteases and amines. Another recently discovered function for serglycin is at the cell surface where it can block effector molecules from reaching their targets at the plasma membrane. Serglycin is mainly expressed by different immune cells such as mast cells, neutrophils and macrophages and expression of serglycin has also recently been noted in some cancer cell types. The expression level seems to affect the behavior of these tumor cells and a high expression correlates with a more aggressive phenotype.

To gain more information about the *in vivo* relevance of serglycin expression during tumorigenesis we are studying the role of this proteoglycan during spontaneous formation of tumors by use of the RIP1-Tag2 mouse model. We have seen that serglycin affects both tumor growth and angiogenesis. We are now further exploring the molecular mechanisms by which serglycin is involved in these processes. Among other things we want to study the impact of an endogenous production of serglycin on cell behavior as opposed to serglycin produced by neighboring cells and how this affects the interaction between tumor cells and tumor stroma cells.

WHAT ARE GLYCOSAMINOGLYCANS GOOD FOR?

Dorothe Spillmann

Our main focus addresses questions how glycosaminoglycans (GAGs), negatively charged, long carbohydrate chains protruding from all cell membranes and intercalated in extracellular matrices, affect diverse cellular processes. GAGs can serve as adhesion sites, co-receptors, stabilizers of molecular interactions, protectors against proteolytic degradation and many more functions. Each cell and tissue produces distinct collections of them with a sophisticated set of enzymes. Absence of GAGs will lead to developmental failure and death during embryogenesis, while structural alteration may be encountered in parallel to disturbed homeostasis in pathologic conditions in adult organisms. Thus, one may wonder whether alterations are cause or result and whether and how GAGs can affect the organism in health and disease.

We recognize GAGs, predominantly heparan sulfate (HS) and chondroitin sulfates/dermatan sulfate (CS/DS), as tuners of molecular interactions at cell surfaces and in the matrix to allow for robust cellular interplay. 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/DS structures modulate cellular behavior and communication, of critical importance to understand the control of physiological and pathological processes in multicellular organisms.

Members of the group during 2014
Anna Eriksson, post doc (until May)
Ulf Lindahl, professor emeritus
Ramesh Babu Namburi, graduate student
Dorothe Spillmann, group leader

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* shared first author

Other publications by Ulf Lindahl:
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GLYCOSAMINOGLYCANS AND CELLULAR PROPERTIES

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. How does the presence of GAGs affect the reception and propagation of stimuli from outside to inside? Are these features affected by how the GAGs are presented, where and how they are localized at the cell surface or in the matrix, attached to a core protein or released as short oligosaccharides by the action of degrading enzymes as for instance used 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 different cellular models to characterize these effects: With different isoforms of the core proteoglycan syndecan expressed in various 'backgrounds' of GAG biosynthesis we study the impact of the core protein and the role of different types of GAG chains on the propagation of extracellular stimuli and resulting cellular activities.

GLYCOSAMINOGLYCANS IN LIMB REGENERATION

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

Many brittle stars, stellate marine invertebrates found in most parts of the world, 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. We use this model to study what role GAGs play for the regenerative capacity of these animals [collaboration with S. Dupont and O. Ortega-Martinez, Kristineberg, GU]. Brittle star species produces a remarkably highly sulfated CS/DS chains correlated with an exceptional limb regeneration capacity and therefore we sought to study the structure/function relationship of these GAGs in the process of limb recovery. After induction of experimental autotomy in arms we characterize GAG production during the regeneration process. During the process GAG sulfation is increased, and conversely, regeneration experiments with interference in biosynthetic sulfation results in a dramatic impairment of arm regeneration by severely affecting cell proliferation. We could demonstrate that several evolutionary conserved morphogens/growth factors interact with these polysaccharides and we therefore aim to identify the corresponding biosynthetic genes in the brittle stars to study their regulation.

MICROBIAL INTERACTION WITH GLYCOSAMINOGLYCANS

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 leading to *e.g.* the development of a treatment approach to reverse symptoms of severe malaria [collaboration with A. Leitgeb, Dilaforette, and M. Wahlgren, KI, Stockholm].
Recently we have shifted focus and started to characterize enzymes used by symbiontic bacteria [collaboration with O. Berteau, INRA, Jouy-en-Josas, France, and M. Rossi, University of Helsinki, Finland]. Sulfatases and hydrolases are among such enzymes that commensal bacteria need for their survival and to digest host GAGs. The main goal to characterize these types of enzymes is to improve our understanding of successful host-microbe symbiosis, to identify potential pathological twists 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 context. 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 of GAGs to further applications and optimize for diverse sample sources. As complementation of our analytic possibilities we collaborate with J. Bergquist and M. Ramström Jonsson at the Dept. of Chemistry, UU, to also set up mass spectrometry-based analytic tools for GAGs. So far we have been able to establish a quick semi-quantitative screening method for large sample numbers that should be a valuable help in deciding further processing of samples before more tedious and time consuming approaches are taken.

On collaborative basis we isolate and characterize GAGs from a wide spectrum of sources for different projects.

MEDICAL PROTEIN CHEMISTRY

Per Jemth, Birgitta Tomkinson, Pia Ek, Leif Andersson

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 fundamental research as well as teaching on both enzymes and non-catalytic proteins. 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. 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 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. Finally, Leif Andersson, professor in functional genomics at IMBIM uses state-of-the-art proteomics to follow up findings from their genomic work.

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 γ-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 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. 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.

The mammalian 14-kDa phosphohistidine phosphatase, also denominated PHPT1, which we found by probing pig liver extracts with a phosphohistidine-containing peptide, has been further investigated. 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 was the ubiquitous expression of the PHPT1-protein and its high expression in continuously dividing epithelial cells.

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, besides the use for studies of PHPT1 and histidine kinases, be used for studies of phosphorylation and dephosphorylation of other acid labile highly basic phosphocompounds in search for proteins that are responsible for these activities. We have phosphorylated histone H1, 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.

Histone H1 does not contain histidine and we found that lysine was the amino acid that was phosphorylated and dephosphorylated in this protein. Chemically phosphorylated polylysine is also a target for PHPT1. This raises questions about the specificity of PHPT1 as well as of its nomenclature. Dephosphorylation of histone H1 by PHPT1 is interesting, since phospholysine has been detected in histone H1 in vivo. The finding opens for future investigation into the role of PHPT1 in regulatory phosphorylation of lysine residues.

An ectopic expression of PHPT1 transcript variant 6 in HeLa cells was undertaken in order to get insight into the possible function of the variant transcripts encoded at the PHPT1 locus. The specific degradation of the PHPT1 splice variant indicates that at least for the PHPT1 protein, the quality control and the self-guarding of the cellular system works at two levels, first at the RNA level, aberrant transcripts are degraded by the non-sense mediated mRNA decay pathway, and the small amount of proteins that are formed will be degraded by the proteasome.

Members of the group during 2014
Pia Ek, Professor em
Örjan Zetterqvist, Professor em

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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 fundamental questions. These model systems are small protein domains from modular proteins, with special focus on intrinsically disordered protein domains. The lab is also running a project on proteins from human papillomavirus, in particular the E6 protein. 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 protein is a so-called oncogene, and a major culprit in the carcinogenesis. We want to inhibit its interaction with cellular proteins with the long-term goal of treating persistent infection.

Members of the group during 2014
Andreas Karlsson, PhD student
Emma Åberg, PhD student
Eva Andersson, research assistant
Fulvio Saccoccia, postdoc
Greta Hultqvist, postdoc
Gustav Sundell, MSc student
Jakob Dogan, postdoc
Mikael Malmqvist, postdoc
Per Jemth, Associate professor

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Agencies that support the work
The Swedish Research Council (M and NT), The Cancer Society.

PROTEINS: FOLDING, STABILITY, INTERACTIONS AND ALLOSTERY

Proteins govern all of life's chemical reactions and they generally do so by first folding into precise three dimensional (3D) structures dictated by their amino acid sequences. But, to great surprise for most scientists, we have learned during the last 15 years that as much as 25% of our proteome is not folded, instead these proteins are disordered. Such intrinsically disordered proteins (IDPs) lack, in part or completely, a well-defined 3D structure. There is now a tremendous interest in understanding structure, function and dynamics of IDPs. However, despite this intense interest of the last couple of years, there is still a marked paucity of experimental data regarding the many aspects of how this disorder influences the function of IDPs and how functional disorder has evolved.

We address these questions using a combination of biophysics, protein engineering and phylogenetic methods. We use different model systems, but up to now most work has been done on two domains from transcriptional co-regulators: "activator domain from thyroid hormone and retinoid receptors" (ACTR) and "nuclear co-activator binding domain of CREB binding protein" (NCBD).

HUMAN PAPILLOMAVIRUS AND CANCER: DESIGN OF A PROTEIN DRUG

The role of human papillomavirus (HPV) in cervical cancer was demonstrated by Harald Zur Hausen who was awarded the Nobel prize in physiology or medicine in 2008. It is now clear that other cancers are also caused by HPV. HPVs are classically divided into two groups: “low risk” and “high risk”. The “high risk” is based on prevalence ratio in cancer, with HPV16 being the most common cause of cervical cancer. 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 the HPV E6 and cellular targets and how this can be utilized to design a protein drug. 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 2014
Birgitta Tomkinson, professor
Jarmila Nahalkova, Ph. D. Researcher

Project workers during 2014
Amanda Abou-Hanna, "Tripeptidyl-peptidase II and the proteasome in acute and chronic myelogenous leukemia: looking for a difference that may explain tumour development."
Matilda Widerström: Tripeptidyl-peptidase II: studies of an interesting enzyme that takes part in intracellular protein turnover (SOFOSKO, part 1).

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Agencies that support the work
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CHARACTERIZATION OF TRIPEPTIDYL-PEPTIDASE II AND INVESTIGATION OF STRUCTURE AND FUNCTION OF TPP II

Birgitta Tomkinson

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 endopeptidase activity of the enzyme has been investigated. This activity is very slow compared to the exopeptidase activity (i.e. the release of tripeptides). Furthermore, the pH-dependence of TPP II from three species with two different substrates was investigated. The results have given some insights into the structure of the active site, and have been expanded with experiments using enzyme variants with point mutations. Various collaborations have also been instigated, exploiting the current knowledge of structure-function relationships for TPP II. Thus, in collaboration with Eric Reits, University of Amsterdam, a specific irreversible inhibitor of TPP II has been characterized and in collaboration with Alexander Zimprich, Medical University of Vienna, a mutant enzyme with a potential role in a neurodegenerative disease has been partly characterized. This work will be continued and investigations aimed at examining the oligomerization of TPP II and if this is a way of regulating enzyme activity *in vivo* are also planned.

TPP II AND CANCER

Amanda Abou-Hanna, Matilda Widerström, 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 used for comparison of results. The different methods have been compared to determine if the amount of mRNA, protein or active enzyme is correlated to tumour malignancy. In a pilot experiment, the amount of TPP II was quantified in blood samples from healthy individuals and patients with different haematological diagnoses. These results will now be used as a starting point for an investigation with a lager number of patient samples.

Interactions between TPP II and the proteasome is also investigated, in order to determine if the increased amounts of TPP II is a secondary effect, for example as a result of decreased degradation or impaired proteasome-function.

PROTEIN-PROTEIN INTERACTIONS OF TPP II

Jarmila Nahalkova

TPP II has, beside its proteolytic function downstream of the ubiquitin-proteasome system, also a potential regulatory effect on cell cycle, apoptosis and senescence.

To investigate the mechanisms of this regulatory effect of TPP II, a protein-protein interaction study was performed to identify the involvement of TPP II in known signalling pathways of human cells. The results of co-immunoprecipitation assays (co-IP) and/or Proximity Ligation Assays (PLA) showed that TPP II interacted physically with the tumour suppressor MYBBP1A, a protein having an activating effect on p53, and the cell cycle regulator CDK2. A mutual protein-protein interaction was also detected between MYBBP1A and CDK2 by combining of co-IP with LC-MS/MS identification of eluted proteins. PLA using HEK293 cells overexpressing TPP II showed that the interaction of TPP II with the tumour suppressor MYBBP1A was TPP II dependent and the interaction in cytoplasm was reversible under serum-free cell growth conditions by the specific inhibitor of TPP II, butabindide, but not in the nucleus. The interacting proteins studied: TPP II, MYBBP1A and CDK2 have cellular functions in protein degradation, tumour suppression, regulation of the cell cycle and apoptosis and they have been previously suggested as targets for development of tumour suppressing agents.

Additional interactions of TPP II were detected with proteins having functions in tumour suppression and neuroprotection, which also have been investigated.

TUMOR BIOLOGY

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

Under the Tumor Biology cluster, IMBIM researchers combine basic and pre-clinical, translational research that is relevant to cancer. Since cancer progression is a complex biological process, the Tumor Biology constellation of scientists focuses mainly on mechanisms by which tumor cells communicate with other cell types within the tumor tissue. By accepting the fundamental role of genetic mutations as causal elements and driving force behind the onset of tumorigenesis, the constellation engages into cell biological problems with the aim at understanding how tumor tissues are organized architecturally, how signaling pathways operate to control tumor cell differentiation and invasiveness, and how specific extracellular matrix components alter the behavior of the tumor tissue including the organization of the tumor vasculature. Through such efforts, the constellation aims at identifying new prognostic tools and therapeutic protocols of clinical significance.

The research projects of the Tumor Biology unit pay attention to the function of infiltrating cell types, such as platelets, and their behavior within the tumor tissue, including their impact on tumor vasculature (*Olsson, Ringvall*). Cell surface receptors, such as integrins, extracellular matrix proteoglycans, such as serglycin, and polymeric assemblies such as collagen and fibrin, generate a complex crosstalk that is responsible for alterations in tumor cell growth, tissue tension and permeability (*Gerwins, Johansson, Ringvall, Rubin*). These and additional signaling interactions drive changes in cell differentiation that involve both tumor cells and tumor stromal cells, including vascular cells, which can generate new cell types within the microenvironment with key functions during progression of the disease (*Moustakas, Olsson, Sundberg*). Finally, the pre-clinical activities of the research unit target specific antigens of the extracellular matrix, such as fibronectin, agents that modulate the differentiation of tumor cells, the tumor blood vessels and the pressure built within the tissue (Gerwins, *Moustakas, Olsson, Rubin*).

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.

Members of the group during 2014
Pär Gerwins, professor, group leader
Peder Fredlund Fuchs, post doc
Ewa Kolosionek, post doc
Femke Heindryckx, post doc
Francois Binet, post doc

Publications 2012 to 2014

1. Kasza Z, Fredlund Fuchs P, Tamm C, Eriksson AS, O'Callaghan P, Heindryckx F, Spillmann D, Larsson E, Le Jan S, Eriksson I, Gerwins P, Kjellén L, Kreuger J. MicroRNA-24 Suppression of N-Deacetylase/N-Sulfotransferase-1 (NDST1) Reduces Endothelial Cell Responsiveness to Vascular Endothelial Growth Factor A (VEGFA). J Biol Chem. Sep 6;288(36):25956-63 (2013).
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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 molecular and physical properties of the surrounding environment, which is important for their migration, differentiation, and proliferation. Integrins are a family of cell adhesion and migration receptors of crucial importance for 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 2014
Staffan Johansson, professor
Xiaofang Cao, postdoc
Deepesh Gupta, PhD student
Ying Huang, postdoc
Siamak Kamranvar, postdoc

Publications 2012 to 2014

1. Riaz, A., Zeller, K.Z., Johansson, S. 2012. "Receptor-specific mechanisms regulate phoshorylation of AKT at Ser473: Role of RICTOR in β1 integrin-mediated cell survival." *PLOSOne* 7(2), e32081
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The Swedish Cancer Society

REGULATION OF SURVIVAL, MIGRARATION, AND CYTOKINESIS BY INTEGRINS

Xiaofang Cao, Deepesh Gupta, Ying Huang, Siamak Kamranvar

A. Adhesion-dependent survival. A central step in integrin-mediated signaling is the activation of PI3 kinase, a key reaction for adhesion-dependent cell survival, actin polymerization, as well as several other signaling pathways. We identified p110α as the catalytic isoform of the PI3 kinase family that is activated by β1 integrins and in detail characterized the downstream pathway to the activation of AKT1/2. Analysis of the requirement of RICTOR, ILK, PAK1/2, FAK, PYK and SRC for AKT Ser473 phosphorylation downstream of β1 integrins, LPA receptors (G protein-coupled), PDGF and EGF receptors (tyrosine-kinases) revealed that the mechanism is more complex than commonly depicted and varies depending on the stimulated receptor. A yet unidentified receptor for the enzyme heparanase was shown to closely cooperate with and depend on integrins for the activation of the PI3 kinase/AKT pathway. We also have indications that TGFβ-induced activation of AKT may depend on integrin-mediated adhesion, and presently we try to characterize these connections.

B. Mechanosignaling. Another important function of integrins is to serve as "mechanoreceptors" for forces acting on the cell. Our results show that the signaling responses to physical force during cell stretching are not the same as the responses to ligand-binding during cell adhesion. Thus, events generally referred to as "integrin signals" are actually composed of separate sets of reactions triggered by different types of integrin stimulation, i.e. integrin clustering by ligands and unfolding of integrin-associated proteins by force. The phosphorylation of ERK1/2 appears to be a particularly stretch-responsive signal, and further studies are required to understand the mechanism for this force-induced ERK activation.

In collaborative studies we analyze the signaling pathways resulting in inflammation and water accumulation in lung alveoli during mechanical ventilation in intensive care situations (in a pig model). In particular, the importance of integrin stimulation and TGFβ activation for the clinical problem is investigated.

Syndecan 4 is known to work together with integrins to organize focal contacts and actin filaments. We investigate the possible role of syndecan 4 in the generation of signals during cell attachment or cell stretching using syndecan 4 knockout MEFs. The absence of syndecan 4 affects several phosphorylation reactions and strongly increases the rate of actin polymerization induced by β1 integrin stimulation. The latter reaction is monitored as lamellipodia protrusion during cell attachment with TIRF microscopy. The mechanisms underlying these observations are investigated.

C. Cytokinesis. Cytokinesis of normal adherent cells requires signals from integrins, and the lack of such signals in detached cells causes 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 divide successfully an increased number of permanently binucleated cells are formed, a feature known to cause aneuploidy and chromosomal instability. We have also shown that the cytokinesis block in suspended cell occurs at a late step, after the recruitment of CEP55 to the midbody. We are presently defining the failing step further and try to identify the integrin signaling pathway regulating this reaction.

D. Adenoviral tumor therapy. 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 concentrates on novel aspects of signal transduction and basic cancer biology. We study 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 aim at explaining how the EMT process contributes to the maintenance of cells that carry tumor-initiating and metastasis-initiating capacities. We have also identified chemical compounds that perturb EMT and try to move our research into more applied medical science.

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

TGFβ and BMP regulate cell growth, differentiation, and tumorigenesis via a group of proteins known as Smads and via protein kinase and GTPase pathways. After activation from the type I and type II TGFβ receptors on the cell surface, the Smads and protein kinases accumulate into the cell nucleus, where they regulate gene expression. TGFβ and BMP signaling have a complex impact on tumorigenesis. The pathways suppress the growth of early-stage tumors by inhibiting cell growth or by prompting cells to undergo apoptosis, but nevertheless drive tumorigenesis in late-stage tumors. We investigate the function and regulation of various TGFβ/BMP-responsive genes by combining functional experiments with genome-wide expression and location analysis in several epithelial cell models. This approach has allowed us to decipher key steps in the genetic programs that mediate tumor suppression or tumor progression in response to TGFβ and BMP.

Members of the group during 2014
Claudia Bellomo, PhD student
Laia Caja, post doc
Mahsa Shahidi Dadras, PhD student
Aristidis Moustakas, professor
Kalliopi Tzavlaki, PhD student

Project workers during 2014
Gad Hatem, project worker (until September 2014)
Prathyusha Naga Pendekanti, project worker (from May to December 2014)
Anna Webb, SOFOSKO summer student (from June to July 2014)

International exchange 2014
Katia Chourlia, visiting project student Erasmus program, Univ. of Thessaloniki, Greece.
Michael Kontakis, visiting undergraduate student, Univ. of Heraklion, Greece.

Satellite group at the Ludwig Institute for Cancer Research (LICR) during 2014
Members of the group
Andries Blokzijl, post-doc (until March 2014)

Jonathon Carthy, post doc
Ulla Engström, technician
Kaoru Kahata, post doc
Constantinos Kolliopoulos, PhD student (50% with Evi Heldin's group)
Varun Maturi, PhD student
Anita Morén, technician
Panagiotis Papoutsoglou, PhD student
E-Jean Tan, PhD student (until February 2014)

Project workers during 2014
Angelos Heldin, project worker (from March to July 2014)
Oskar Idås, project worker (until November 2014)
Simon Olofsson, project worker (from January to June 2014)

International exchange during 2014
Savvas Petanidis, visiting PhD student, Univ. of Thessaloniki, Greece.
Michael Nacif, visiting Master's student, Sadat City University, Egypt.

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REGULATION OF TGFβ/BMP RECEPTOR SIGNALING BY PROTEIN KINASES

Mahsa Shahidi Dadras, Kalliopi Tzavlaki

We are interested in two members of the AMP-regulated kinase (AMPK) family, widely known as being substrates of the master kinase and tumor suppressor LKB1. These are the

salt-inducible kinase (SIK) and the Nuak2 kinase, whose genes are immediate-early targets of TGFβ signaling. We try to uncover the molecular links between TGFβ, BMP and LKB1/AMPK signaling by focusing on mechanisms of receptor function and trafficking. SIK regulates turnover of the TGFβ receptor after ligand binding by cooperating with Smad7 and the Smurf ubiquitin ligases. The new model that we have generated so far for BMP signaling shows that LKB1 negatively regulates the BMP type I receptor ALK2, a process important during Drosophila organogenesis and lung cancer progression. Negative regulation of receptor stability requires the inhibitory Smad7 and formation of a ternary complex between the receptor, Smad7 and LKB1, in which SIK promotes ALK2 ubiquitination.

This work has been partially carried in collaboration with Anita Morén, Drs. Erna Raja and Carl-Henrik Heldin (LICR-Uppsala University), Dr. Patrick Micke (Department of Immunology, Genetics and Pathology, Uppsala University), Dr. George Pyrowolakis (Freiburg University, Germany) and Dr. Ola Söderberg (Department of Genetics and Pathology, Uppsala University).

MOLECULAR MECHANISMS OF EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

Claudia Bellomo, Mahsa Shahidi Dadras, Kalliopi Tzavlaki

EMT is an important process during cancer dissemination and contributes to the generation of cancer stem cells. In our recent work, we analyze the role of LKB1 and SIK kinases in regulating critical aspects of the EMT process, including cell polarity. LKB1 promotes epithelial differentiation, while TGFβ by inducing SIK promotes the mesenchymal transition. We identified new substrates of SIK as proteins that regulate the cytoskeleton and epithelial polarity. Our model suggests that SIK, via phosphorylation, provides signals for proteasomal degradation of its substrates. Inhibitors of the SIK kinase would perturb the EMT response. We also analyze the role of LKB1 in controlling epithelial polarity by knocking out the LKB1 gene in three dimensional, polarized epithelial cell models. In our project under the ITN "IT-Liver", we study liver cancer cells at different stages of differentiation for sensitivity or resistance to a panel of compounds that either affect the differentiation of these cells or cause synthetic lethality together with TGFβ. We characterize new molecular pathways that may mediate such responses of the liver cancer cells and have been focusing on the nuclear receptor superfamily of transcription factors. In addition, we have developed new assays for the analysis of cancer stem cells in hepatocarcinoma using single spheroid cultures in hanging drops and immunocytochemistry of the spheroids.

This work has been partially carried in collaboration with Drs. Jonathon Carthy and Carl-Henrik Heldin (LICR-Uppsala University), Drs. Andrew Shiau and Timothy Gahman (LICR-San Diego, USA), Dr. Martin Stöter (Max Planck Institute, Dresden, Germany), Dr. Steven Dooley (Clinical Medicine at Mannheim, Heidelberg University, Germany), Dr. Isabel Fabregat (Institute for Biomedical Investigations at Bellvitge, Barcelona, Spain), Dr. Wolfgang Mikulits (Medical University of Vienna, Austria) and with Drs. Christer Busch, Patrick Micke and Fredrik Pontén (Department of Immunology, Genetics and Pathology, Uppsala University).

LINKS BETWEEN INVASION AND SELF-RENEWAL OF TUMOR INITIATING CELLS

Laia Caja Puigsubira, Mahsa Shahidi Dadras, Kalliopi Tzavlaki

We analyze the role of BMP signaling in the suppression of stemness of tumor-initiating cells of the brain (glioblastoma multiforme, GBM). Via a genome-wide screen for mRNAs expressed under the control of BMP7, which suppresses GBM tumorigenesis, 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 Snail. Our current work focuses on four axes: a) identification of the feedback loops between BMP, TGFβ signaling and Snail transcription. b) Analysis of Snail target genes that mediate GBM cell invasiveness. c) Analysis of the role of the LKB1 kinase in GBM stem cell self-renewal. d) Analysis of the transcriptional regulation of oligodendrocyte differentiation in the context of GBM.

This work has been partially carried in collaboration with Drs. Jessica Cedervall and Anna-Karin Olsson (Department of Medical Biochemistry and Microbiology, Uppsala University), Drs. Lene Uhrbom, Bengt Westermark and Karin Forsberg-Nilsson (Department of Immunology, Genetics and Pathology, Uppsala University).

SUMMARY OF ACTIVITIES AT THE LICR

We focus on the role of the ribosyl-transferases PARP-1 and PARP-2 and the dePARylating enzyme PARG, 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 TGFβ and BMP signaling. We also attempt to understand how post-translational modifications of Smads may change between normal and cancer cells. Using in situ proximity ligation we can detect the endogenous protein interactions and post-translational modifications. We also analyze the genome-wide location of major EMT transcription factors in breast cancer cells, while analyzing the role of specific long non-coding RNAs on EMT and breast cancer cell stemness. The chromatin regulator high mobility group A2 (HMGA2) protein mediates EMT in response to TGFβ by facilitating the transcriptional induction of the transcription factors Snail and Twist. We have deciphered a new mechanism by which HMGA2 represses the *E-cadherin* gene via DNA methylation on its promoter. We have also identified novel compounds that affect myofibroblast activation in response to TGFβ and analyze the role of nuclear receptors in this process. Finally, in a recent collaborative project we work on the signaling pathway by the TGFβ family member GDF-15 (growth differentiation factor 15).

This work is carried in collaboration with Ulla Engström, Varun Maturi, Anita Morén, Panagiotis Papoutsoglou, Drs. Andries Blokzijl, Jonathon Carthy, Kaoru Kahata and Carl-Henrik Heldin (LICR-Uppsala University), Dr. Ulf Landegren (Department of Genetics and Pathology, Uppsala University), Dr. Michael Hottiger (University of Zurich, Switzerland) and Dr. Takeshi Imamura (Ehime University, Japan).

TUMOR VASCULAR BIOLOGY

Anna-Karin Olsson

Cancer is a systemic disease and mortalities are mainly caused by metastasis, deep vein thrombosis and a general organ failure. These complications all involve a tumor-induced interplay with the vasculature, both in the primary tumor as well as in peripheral organs. We aim to get a better understanding of this tumor-vessel interface, with the ultimate goal to improve current cancer therapy and to reduce adverse events caused by the disease or current treatment strategies. We are also exploring how tumor vessels can be targeted to treat cancer.

A main interest in the lab is the mechanism by which platelets promote angiogenesis, tumor progression and metastasis. Cancer patients commonly display elevated platelet activation and suffer from 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.

We are also interested in the genetic and molecular mechanisms responsible for deregulated blood vessel formation. We have previously identified histidine-rich glycoprotein (HRG) as en endogenous regulator of tumor vascularization. Using various genetic models we address the mechanism(s) of action of this plasma protein.

One of our projects is focused on the development of therapeutic cancer vaccines directed specifically at molecules expressed by tumor vessels. A main advantage of targeting the tumor vessels is that they have not developed the same immune escape mechanisms as the tumor cells. In addition, the vasculature has a more stable genetic composition and is easily accessible for the immune system.

Members of the group during 2014
Jessica Cedervall, post-doc
Julia Femel, graduate student/post-doc
Anna-Karin Olsson, Assoc Prof, group leader
Falk Saupe, post-doc
Yanyu Zhang, graduate student

Project students during 2014
Maja Eriksson, UGSBR, Uppsala University

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The Medical Faculty, Uppsala University

SYSTEMIC EFFECTS OF CANCER

Jessica Cedervall, Yanyu Zhang

Cancer mortality is mainly connected to systemic effects induced by the primary tumor such as metastasis, deep vein thrombosis and a general organ failure. Studies of tumor-induced effects on distant organs have primarily focused on tissues that represent metastatic sites. Surprisingly little is however known about the situation in organs that are not targets for metastasis or affected by the primary tumor. For obvious reasons human biopsy material from these tissues is rare and mouse models therefore become important tools for such investigations. Using the two orthotopic and metastasizing models RIP1-Tag2 for insulinoma and MMTV-PyMT for breast cancer, both believed to closely resemble disease development in the human situation, we have found a dramatic reduction in the vascular function in kidney and heart compared to healthy littermates of the same genetic background. We have identified the neutrophil and its ability to form neutrophil extracellular traps (NETs) as a major component in the reduced vascular function in peripheral organs in individuals with cancer. Moreover, our data indicate that systemic inflammation and endothelial activation takes place in peripheral organs as a consequence of cancer. Our current activities aim to identify the tumor-derived molecules and mechanisms involved in this process.

THE ROLE OF PLATELETS IN TUMOR VASCULARIZATION, EPITHELIAL-MESENCHYMAL TRANSITION AND METASTASIS

Jessica Cedervall, Yanyu Zhang

Platelets are central players in maintaining hemostasis of the blood. At sites of blood vessel injury, platelets are activated to induce blood coagulation and form aggregates at the site of the damaged endothelium to prevent hemorrhage and thereby protect us from fatal bleedings. Besides their role in hemostasis, platelets have been shown to contribute to non-hemostatic processes such as wound healing, immunity, angiogenesis, cardiovascular disease, epithelial-to-mesenchymal transition and metastasis. Our research is focused on the mechanisms by which platelets regulate these processes, with a special focus on the role of HRG (described below). Using *in vitro* as well as *in vivo* assays we have identified a number of molecules potentially involved in the platelet-induced effects in the pre-tumorigenic environment.

HISTIDINE-RICH GLYCOPROTEIN IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

Jessica Cedervall, Yanyu Zhang

Histidine-rich glycoprotein (HRG; alternatively, HRGP/HPRG) is a heparin-binding plasma protein that 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. 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. Moreover, epithelial-mesenchymal transition (EMT) as well as metastasis is accelerated in HRG-deficient mice. Mice lacking HRG display enhanced coagulation and increased platelet activation and we have found that several features of the accelerated tumorigenesis in HRG-deficient mice are mediated by platelets.

TARGETING TUMOR VESSELS BY THERAPEUTIC VACCINATION

Julia Femel, Falk Saupe, Maja Eriksson

One of the major success stories in human and veterinary medicine during the past 100-150 years is vaccines targeting various infectious diseases. Vaccines have together with antibiotics likely been more important for human and companion animal health than any other part of human or veterinary medicine. Due to the success of vaccines the interest in using vaccine technology for the treatment of non-infectious diseases like allergies, autoimmunity and cancer is increasing. Therapeutic vaccination targeting self-molecules could provide a cost-efficient alternative to monoclonal antibody-based therapies for cancer and various inflammatory diseases. The development of cancer vaccines has, however, so far not been successful enough to qualify as a standard therapy in the clinic. The reason for this is probably multifaceted, but one complicating factor is that tumor cells have developed strategies to escape recognition by the immune system. Antigens specifically expressed by the tumor vasculature can therefore provide alternative targets.

A small number of molecules have been identified as specifically expressed by neoplastic vasculature, either by the endothelial cells or by the surrounding matrix. The reported lack of expression of these molecules in healthy tissue renders them highly interesting for targeted cancer therapies. Examples are the alternatively spliced extra domains-A and –B (ED-A and ED-B) of fibronectin (FN). These extra-domains of FN are expressed during vasculogenesis in the embryo but essentially undetectable under normal conditions in the adult. Both ED-A and ED-B are highly expressed around angiogenic vasculature in various tumor types and show a strong conservation between species. We have in two recent publications shown that it is possible to compromise tumor vessel function and as a consequence suppress growth of aggressive pre-clinical tumors by immunization against ED-A or ED-B, both in a prophylactic and a therapeutic setting. Furthermore, we found that in mice with anti-ED-A antibodies the number of metastases was reduced in a transgenic model of metastatic mammary carcinoma. These data suggest that tumor vascular antigens are highly interesting candidates for development of therapeutic vaccines targeting solid tumors. Currently we are working on design of multi-targeting vaccines to achieve a broad coverage of many tumor types simultaneously and to further increase the efficacy of the vaccine.

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 maintenance of 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 found a correlation between inflammatory processes and the architecture of the collagen network of the stroma during regulation of IFP.

Members of the group during 2014
Vahid Reyhani, PhD, postdoc
Lars Rask, PhD, professor in Medical Biochemistry
Kristofer Rubin, PhD, professor in Connective Tissue Biochemistry (leave of absence)

Publications 2012 to 2014

1. Friman, T., Gustafsson, R., Stuhr, L.B., Chidiac, J., Heldin, N.-E., Reed, R.K., Oldberg, Å. and Rubin, K (2012) Increased fibrosis and interstitial fluid pressure in two different types of syngeneic murine carcinoma grown in integrin □3-subunit deficient mice. *PLoS ONE* 7(3): e34082. doi:10.1371/journal.pone.0034082
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SIGNALING PATHWAYS INVOLVED IN BOTH INTEGRIN- AND PDGF-DERIVED COLLAGEN GEL CONTRACTION

Vahid Reyhani

Cell-mediated matrix contraction plays a crucial role in regulation and maintenance of the IFP. This contraction process 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 collagenous 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 presence of RGD-containing fibrous ligands such as fibrin. Presently the

work is concentrated on the signaling events downstream of PDGF-Rβ and integrins, during cell-mediated matrix contraction. More specifically, to study signaling pathways involved in generation of cellular contractile forces, downstream of PDGF-Rβ, collagen-binding β_1 integrins, and $\alpha_V\beta_3$ integrin.

POTENTIAL ROLE OF FIBRIN IN CLEARANCE OF EDEMA AND TISSUE HOMEOSTASIS

Vahid Reyhani

The functional significance of fibrin deposits typically observed in inflammatory sites, carcinomas and in healing wounds is not fully understood. Recently we described a novel biological significance of fibrin in such pathologies, where the collagen-binding β_1 integrin signaling is impaired. The extravasated fibrin provides an interface between the collagenous matrix and cells to allow them to re-exert contractile forces on the matrix. This process can potentially be part of an edema clearance program *in vivo*. We also, for the first time, characterized the direct binding of fibrin to collagen type I, and showed that this binding plays an essential role in the stability of the interface fibrin network. Currently our focus is to investigate other possible impacts of this interaction, for example whether this binding protects collagenous fibers of the ECM from peptidase degradation during inflammation, as well as, whether fibrin can trigger assembly of new collagenous matrix at inflammatory lesions.

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.

Members of the group during 2014
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 2012 to 2014
1. Rodriguez A, Friman T, Gustavsson R, Kowanetz M, van Wieringen T and Sundberg C. (2013) 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. Jun;182(6):2132-46.

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.

MEDICAL MICROBIOLOGY

IMMUNOLOGY

Birgitta Heyman, Jenny Hallgren Martinson

The cellular and humoral components of the immune system are crucial in our defense against foreign microorganisms. The central themes 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 and 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 of the group during 2014

Anna Sörman (f. Bergman), PhD student
Joakim Bergström, PhD student
Zhoujie Ding, PhD student
Birgitta Heyman, professor, group leader
Annika Westin, technician
Hui Xu, PhD student
Lu Zhang, PhD student

Publications 2012 to 2014

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MECHANISMS FOR COMPLEMENT-MEDIATED REGULATION OF IMMUNE RESPONSES

Anna Sörman, Zhoujie Ding, Lu Zhang, Hui Xu, 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 the 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. We have shown that both antibody classes induce enhanced antibody-, but not T cell-responses, enhanced germinal center reaction and are dependent on CR1/2 expressed both on B cells and follicular dendritic cells. IgG3 causes antigen to be deposited in splenic follicles. We will now investigate the effects of induction of immunological memory.

MECHANISMS FOR IgG-MEDIATED SUPPRESSION OF IMMUNE RESPONSES

Joakim Bergström, Hui Xu, 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, on the development of germinal centers in the spleen and on development of immunological memory and longlived plasma cells. The role of complement activation and Fc-receptors in these processes will be investigated.

MECHANISMS FOR IgE-MEDIATED ENHANCEMENT OF IMMUNE RESPONSES

Zhoujie Ding, Hui Xu, Birgitta Heyman

IgE passively administered to mice together with its specific antigen will bind to circulating B cells via their low affinity receptor for IgE, CD23. After 30 minutes the antigen has been transported to the areas in the spleen where the fine tuning of antibody responses takes place, the follicles. There it is delivered to dendritic cells which internalize and present the antigen to T cells which in turn help B cells to produce antibodies. The result is a potent T cell proliferation followed by a severel 100-fold enhanced antibody response. We will now try to detect the antigen in vivo in various cell types using flow cytometry. Mice will be immunized with antigen conjugated to fluorophores that can be detected intracellularly. After various times, the cells from the spleen are analyzed for antigen content. In addition, we will investigate which subgroup of dendritic cells that actually present the peptides to T cells using cell sorting, in vitro proliferation assays as well as confocal microscopy.

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 degranulates mast cells and release proinflammatory mediators such as tryptase and histamine. Mast cells mature in tissues from committed mast cell progenitors that are rare but can be quantified by limiting dilution assay or multi-colour flow cytometry. The mouse lung contains few mast cell progenitors, but allergic inflammation or respiratory infection 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 asthmatic patients. We study the mechanisms behind the mast cell increment in the lung and the role of mast cells and their progenitors in allergic asthma and respiratory infections.

Members of the group during 2014
Joakim Dahlin, Post-Doc
Jenny Hallgren Martinsson, Associate Professor, Group leader
Erika Mendez Enriquez, Post-Doc (From August 2014)
Maya Salomonsson, Project assistant (From September 2014)
Behdad Zarnegar, PhD student
Annika Westin, laboratory engineer

Project workers during 2014
Viktor Persson, Pontus Öhlund, Malin Castelius

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HUMAN MAST CELL PROGENITORS

Joakim Dahlin, Jenny Hallgren Martinsson

This project is aimed at identifying and quantifying human mast cell progenitors with flow cytometry. The development of mast cells from early progenitors to a committed mast cell progenitor has been studied in the mouse. Recently, we identified a committed mast cell progenitor in mouse blood. However, nobody has yet found a committed human mast cell progenitor. We hypothesized that we will identify a committed mast cell progenitor population in human blood by evaluating the mast cell potential in sorted cell populations from human blood.

WHAT ARE THE MECHANISMS BEHIND THE INCREASE IN LUNG MAST CELL PROGENITORS SEEN IN ALLERGIC ASTHMA AND RESPIRATORY VIRUS INFECTIONS?

Behdad Zarnegar, Annika Westin, Joakim Dahlin, Kjell-Olov Grönvik, Jenny Hallgren Martinsson

The increase in lung mast cell progenitor in an experimental asthma model is rapid and can be inhibited by antibody blocking or genetic deletion of molecules involved in endothelial transmigration. This suggests that the increase in mast cell progenitors is largely due to recruitment. Preliminary results suggest that infection of mice with influenza virus also causes increased numbers of mast cell progenitors in the lung but since this occurs around one week after virus inoculation there is a possibility that mast cell progenitors while likely being recruited from the blood also divide in situ. We are currently investigating how much of the increase in mast cell progenitor numbers in the lung that is due to recruitment and how much that is caused by cell division in situ in these two experimental disease models.

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

Annika Westin, Behdad Zarnegar, Erika Mendez Enriquez, Kjell-Olov Grönvik, Jenny Hallgren Martinsson

We hypothesized that mast cells are involved in the exacerbations of allergic asthma seen after respiratory infections via a combination of mechanisms: 1) Virus-infections 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 by antigens 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 activate pattern recognition receptors. We are using experimental models of allergic asthma and respiratory infections to mimic viral induced exacerbations. By quantifying the degree of mast cell activation and mast cell numbers, we aim to find out if mast cells are more activated by the combination of virus infection and allergic asthma or if the worsening are due to mast cells that are equally activated but higher in numbers.

THE ROLE OF MAST CELLS IN ALLERGIC AIRWAY INFLAMMATION

Erika Mendez Enriquez, Behdad Zarnegar, Jenny Hallgren Martinsson

Previous studies of the role of mast cells using mouse models of allergic airway inflammation have been performed in different mouse strains with mutations in the White-spotting locus (W), which codes for the stem cell factor receptor, c-kit. Since stem cell factor is a critical growth and maturation factor for mast cells, these strains lack mast cells. However, stem cell factor is also a critical growth factor during the early haematopoiesis for normal development of other lineages making these strains having other deficiencies. In this project we aim to validate the findings associated with mast cells observed in c-kit dependent models using a new kit-independent model of mast cell deficiency. In collaboration with Thorsten Feyerabend and Hans-Reimer Rodewald we use their new mast cell deficient mice strain. This strain has a targeted insertion of Cre-recombinase into the mast cell carboxypeptidase A3 locus (CPA3-Cre mice), which induces a deletion of mast cells by a genotoxic Trp53-dependent mechanism. To model allergic airway inflammation, we use house dust mite, a common allergen that also induces human asthma.

MOLECULAR BACTERIOLOGY

Dan Andersson, Diarmaid Hughes, Linus Sandegren, Göte Swedberg

The area of molecular bacteriology at IMBIM is made up of four 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 AND DYNAMICS OF BACTERIAL ADAPTATION AND EVOLUTION

Dan Andersson

We study the mechanisms and dynamics of molecular evolution in bacteria and are interested in 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 (Salmonella enterica, Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus and Mycobacterium 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 S. enterica and E. coli as model systems to: 1. Examine the role of gene amplification in adaptive responses to antimicrobial drugs and in the evolution of novel genes. 2. Examine the mechanisms and physiological effects of deletion formation. 3. Examine the functional role of ribosomal proteins and the mechanisms by which their absence can be genetically compensated. 4. Examine the fitness effects and constraints on horizontal gene transfer. 5. Examine the distribution of fitness effects of random mutations in different types of proteins and its impact on adaptive evolution. 6. Examine the mechanisms by which synonymous mutations can affect bacterial fitness and growth rates. 8. Experimentally study de novo evolution of new genes.

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 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. Identify mechanisms that confer resistance to antimicrobial peptides and determine the impact of these mechanisms on bacterial fitness and virulence. 5. 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 2014
Dan I. Andersson, Professor
Ulrika Lustig, Research engineer
Karin Hjort, Researcher
Peter Lind, Researcher
Herve Nicoloff, Researcher
Joakim Näsvall, Researcher

Marlen Adler, Postdoc (with Dr. Linus Sandegren)
Jessica Kubicek Sutherland, Postdoc
Omar Warsi, Postdoc
Erik Gullberg, PhD student/postdoc
Anna Knöppel, PhD student
Marius Linkevicius, PhD student
Hava Lofton, PhD student
Erik Lundin, PhD student
Sohaib Malik, PhD student (shared with Dr. Ulf Göransson)
Annika Söderholm, PhD student (shared with Dr. Maria Selmer)
Elisabeth Thulin, PhD student

Project students and visitors
Marie Nykvist
Roderich Römhild
Abeer Hegazy
Usama Hegazy

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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 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. Metals of interest are arsenic, a component in poultry growth promoters and in pesticides, and silver, an antimicrobial used 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 these genes may enable a plasmid to be maintained in a population that is exposed to even very low levels of metals. The plasmid investigated is pUUH239.2 that was isolated from Uppsala University Hospital. It carries resistance genes to several antibiotics, and in addition it harbors arsenic and silver resistance operons. The competitive ability of the plasmid strain in a metal-containing environment is measured by flow cytometry, using the fluorescent tags blue fluorescent protein and yellow fluorescent protein for detection of the strains. It was found that both arsenic and copper levels well below the minimum inhibitory concentration (MIC) confers positive selection on the plasmid. Furthermore, mixtures of sub MIC levels of heavy metals and antibiotics exert a combination effect where each substance in the mixture contributes to plasmid selection, showing that combinations of small amounts of metals and antibiotics can select for a multidrug resistance plasmid.

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 performing 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 investigate how low levels of antibiotics affect the selection, conjugation and maintenance of conjugative resistance plasmids in bacterial populations. Since conjugative resistance plasmids frequently carry genes conferring resistance to heavy metals as well as antibiotics, we study the selective effects of combinations of sub-inhibitory concentrations of antibiotics and heavy metals.

We also investigate 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.

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

Karin Hjort

Bacterial populations can develop de novo resistance against antibiotics at sub-MIC (minimal inhibitory concentration) levels. This may generate a challenging environmental problem since sewage water, lake water and farm run-off contain low levels of antibiotics. The measured amount for specific antibiotics in these environments are in some instances within the range known to increase the frequency of pre-existing antibiotic resistant bacteria and select for de novo resistance. In this project, we study the antibiotic colistin, clinically used for multiresistant bacteria such as extended-spectrum β-lactamases (ESBL) producing bacteria. The focus of this project is to examine colistin's ability to enrich for de novo resistant mutants of E. coli and S. enterica during serial passage at sub-MIC levels. Our results shows that resistant populations of E. coli and S. enterica are selected at sub-MIC concentrations of colistin and that the level of antibiotic resistance increased during the cycling experiment. All the S. enterica isolates showed heteroresistance, mixed populations of susceptible and resistant phenotypes. The frequency of resistant bacteria was unstable and increased with antibiotic selection. Whole genome sequencing of colistin resistant strains showed different resistance mutations in E. coli and S. enterica. For E. coli the resistant strains contained mutations in the pmrA and pmrB genes part of the two-component system involved in sensing low pH, Al^{3+} and Fe^{3+}. Mutations in these genes are known to generate resistance in both E. coli and S. enterica at high concentrations of colistin. In the colistin resistant S. enterica genomes no known resistance genes were mutated. Instead genes (arnT

and prmD) known to be involved in colistin resistance were amplified. With an amplification of the pmrD genome region under the regulation of an antibiotic cassette, the increased copies of the pmrD gene were directly proportional to an increase in the colistin resistant population frequency. A knockout of the pmrD gene abolished the resistance completely. These experiments will give us a better understanding of the spread of antibiotic resistance at sub-MIC levels of antibiotics in the environment and the implications of heteroresistance due to gene amplification/segregation. Our data suggests that sub-MIC growth result in the selection for novel types of resistance mutations, different from those that are found at lethal drug concentrations.

ESTABLISHING AN IN VIVO METHOD FOR COMPETITION ASSAYS OF ANTIBIOTIC RESISTANT MUTANTS

Karin Hjort

Most experimental setups to study evolution and fitness cost of antibiotic resistant bacteria involve 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 common 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 that can increase the frequency of antibiotic resistant bacteria and also select for de novo generated mutants. Bacterial fitness cost can be analyzed by competition experiments between resistant mutants and wild type bacteria where the changes of bacterial populations are determined with fluorescently labeled bacterial populations in a flow cytometer. Our preliminary data indicates that wild type E. coli can grow with a reasonable generation time and population size in autoclaved and filtered sewage water from the inlet of Kungsängen 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.

EVOLUTION OF COMPLEMENT RESISTANCE IN BORRELIA

Jon Jerlström-Hultqvist

This project will investigate the potential of Borrelia strains to resist complement mediated lysis by the mechanism described in the innovation-amplification-divergence (IAD) model. The IAD model predicts the creation of new genes often happens by selection on an already weak preexisting promiscuous activity (the innovation) in a protein. If the weak activity becomes under selection it may initially be compensated by amplification of the gene. Amplification creates a larger mutational target and might lead of new variants by accumulation of mutations. Mutations that improve the weak activity will lead to the collapse of the amplified gene array and the birth of a novel gene. Predictions of this model include that evidence of positive selection should be found in the newly evolved gene copy. The Pfam54 gene family of Borrelia is interesting because there is evidence of positive selection acting on certain amino acid positions and only some family members have the capacity to

bind to human fH and FHL-1 exclusively. The non-monophyly of hfH and FHL-1 binding among CRASP-1 homologs points to accidental promiscuous activity that has arisen in some members of this family.
Most B. garinii isolates have limited to no serum resistance. In line with this the BgCRASP-1 proteins bind only weakly to FHL-1 and (not at all) to fH. Further, the expression of BbCRASP-1 in serum sensitive B. garinii has been shown to endow the cells with complement resistance. Non-immune human serum (NHS) will be applied to select a serum sensitive B. garinii isolate to acquire the ability to survive the serum challenge by amplification of weak binding activity of its BgCRASP-1 homologs and eventual divergence of the amplified gene copies within the amplified array will be determined by sequencing the evolved strains.

FISHING FOR GENES WITH PROMISCUOUS ACTIVITIES

Jon Jerlström-Hultqvist

The evolution of new genes is a central question in biology that might help to explain the large variability observed in living organisms today. It is clear that new genes may evolve along many different routes, in bacteria gene duplication and horizontal gene transfer appears to be especially important mechanisms. The genetic underpinnings that lead to the establishment of novel genes is incompletely understood but may involve incremental improvement of protein function through selective pressures. The existence of weak enzymatic activities provides a mechanism whereby new genes may evolve through innovation (the weak activity), amplification and divergence (IAD) model where positive selection drives the generation of novel gene variants with increased activities. In this project we aim to screen for novel genes conferring resistance to antibiotics or metabolic innovation through weak promiscuous activities. We have conducted screens using an E. coli proteome overexpression library to judge the potential of endogenous genes. In addition we will investigate the evolutionary potential of phage proteins by employing metagenomic phage libraries from several different environments (coral, riverine, pond, mucus) to simulate the potential from horizontal gene transfer. Metabolic innovation screens have been performed using single-gene knock-out auxotrophs and the phage metagenomics libraries. We have identified candidate genes and metagenomics gene fragments that increase the resistance to antibiotics and rescue of auxotrophy . The mechanism and adaptive potential behind the observed phenotypes is now under study.

STRAIN-SPECIFICITY AND EPISTATIS OF DIFFERENT RESISTANCE MECHANISMS

Michael Knopp

The phenotypic expression of resistance mechanisms is thought to be largely independent of the genetic background. To investigate the strain specificity of antibiotic resistance mechanisms we constructed five characterized resistance mutations (*rpsL*, *rpoB*, *fmt*, *fusA* and *gyrA*) in four different strains of *Salmonella enterica*. Our results show that the phenotypic expression of the five investigated resistance mutations is completely independent of the strain context. The effect on fitness and resistance is constant in all four investigated strains. Another important aspect of the influence of the genetic background on the phenotype of a

resistance mutation is potential epistatic interactions between the phenotypic expression of resistance mechanisms. Two mutations have negative, neutral or positive epistasis, if the fitness of a strain carrying both mutations is lower, equal or higher than the product of the fitness of the two individual mutants. In the case of positive epistasis the double mutant has a higher fitness than expected. This could lead to a selection against the loss of resistance in a multi-resistant strain, because the loss of any resistance mechanism would lower the fitness of the bacterium. To test epistatic interactions between the five investigated resistance mutations we constructed all possible combinations of these mutations in *S. enterica* serovar typhimurium LT2. Assuming all combinations are viable this would yield five single, ten double, ten triple, five quadruple and one quintuple mutant. The so far constructed combinations show a pervasive additive effect. This contradicts recent publications, which report strong epistatic interactions among the phenotypic expression of resistance mutations. We are investigating the cause of this discrepancy, which is possibly due to the use of different resistance mutations, different organisms or a different method of fitness determination.

RAPID AND EFFICIENT COMPENSATION OF PORIN-LOSS IN ESCHERICHIA COLI

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. Many studies have focused on the compensation of fitness costs that are based on resistance mechanisms due to target alterations and where typically the mechanism of compensation involves point mutations that restore function of the affected target molecule or process. Here we investigate how bacteria can compensate the adverse effects that are often accompanied by resistance due to a decreased import of antibiotics. Using long-term evolution experiments we were able to minimize the associated fitness cost of the loss of two major porins in E. coli: OmpC and OmpF. By regular 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. Compensation of OmpC/OmpF loss in E. coli follows two pathways: Mutations in hfq restore the growth rate and maintain the resistance to ertapenem and meropenem supposedly by upregulation of the alternative porin ChiP. Mutations in the regulator PhoR, causing a constitutive activation of the PHO-regulon, restore the growth rate but completely abolished the resistance. Compensation via PHO-activation is possibly due to an increased expression of the porin PhoE. For both pathways compensation of fitness costs is very rapid and efficient and involve the induction of alternative porins that compensate for the loss of OmpC and OmpF.

DE NOVO GENES FROM RANDOM SEQUENCES

Michael Knopp, Hervé Nicoloff, Jon Jerlström-Hultqvist

De novo gene birth results from random nucleotide sequences acquiring transcription and translation abilities. However, the vast size of the sequence landscape makes it highly improbable to find a specific biological function in a random sequence of amino acids. Consequently, appearance of the first biological functions on earth would have resulted from extremely improbable events. One way to circumvent this dilemma could be if the same function can be found in many different sequences or structures but that life, which must have originated from a relatively small set of de novo genes, would only use a small subset of those sequences/structures for any given function or activity. To test this hypothesis, we intend to experimentally select for biological functions encoded by random peptides. For this, we have designed six libraries of random nucleotide sequences encoding random peptides differing in size and composition. By varying amino acid content, we intend to sample specific regions of the sequence landscape (e.g. sampling for sequences depleted in hydrophobic residues to favor disordered peptides, or sampling for sequences enriched in "primordial" amino acids). Random peptides potentially encoding specific biological activities will be positively selected for i) rescue of auxotrophy, ii) rescue of thermosensitivity, iii) antibiotic resistance, iv) utilization of new carbon sources and v) replacement of ribosomal proteins. Further analysis will be performed to determine the exact activity encoded by the random peptides selected.

GENETIC COMPENSATION OF THE FITNESS COSTS OF SYNONYMOUS MUTATIONS

Anna Knöppel, Joakim Näsvall

Although synonymous mutations do not change the sequence of the polypeptide, an increasing number of studies have shown that they often confer a cost. In this study we have investigated why four synonymous mutants in rpsT, encoding ribosomal protein S20, in Salmonella enterica are costly, and how this cost can be compensated for. The synonymous mutants were found to have low levels of S20 due to reduced translation of the rpsT mRNA. Previous studies have shown that 30S ribosomal subunits lacking S20 are impaired in mRNA binding and docking of the 30S initiation complex to the 50S subunit. In an adaptive evolution experiment, these impairments were compensated by either (i) up regulation of S20 though increased gene dosage (duplications), increased transcription of rpsT (rpoD mutation), or, increased translation from the rpsT transcript (intragenic rpsT mutations), or alternatively, by (ii) down regulating ribosome synthesis through mutations affecting the global regulator Fis. We suggest that the cost of these synonymous mutations is due to production of a dysfunctional and potentially toxic subpopulation of 30S subunits lacking S20, and that this cost can be compensated by mutations that brings the ratio S20:ribosomes closer to 1:1 by either increasing the rate of S20 synthesis or reducing the rate of ribosome synthesis to match the low levels of S20.

MINOR FITNESS COSTS IN AN EXPERIMENTAL MODEL OF HORIZONTAL GENE TRANSFER IN BACTERIA

Anna Knöppel, Joakim Näsvall, Ulrika Lustig

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 ecological and 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 varying in size from 0.45 to 5 kb of Bacteroides fragilis, Proteus mirabilis and human intestinal phage were inserted into a neutral position of the Salmonella typhimurium chromosome and the fitness costs of the inserts determined and correlated to size and origin of insert, GC content, gene type, and the expression level of inserts. We found that 8 inserts were deleterious and ninety inserts did not have any detectable fitness effects. When inducing transcription from a P_{BAD} promoter located at one end of the inserts, 16 transfers were deleterious and 82 did not have any detectable fitness effects. In conclusion, a major fraction of the inserts had minor effects on fitness implying that extra DNA transferred by HGT, even though it does not confer an immediate selective advantage, could be maintained at transfer-selection balance and serve as raw material for the evolution of novel beneficial functions. We could not detect any correlation between fitness costs and origin of insert, GC content or gene type. The fitness costs, however, correlated positively with expression level and the number of inserts with detectable deleterious effects increased under inducing conditions.

HORIZONTAL TRANSFER OF ANTIBIOTIC RESISTANCE GENES IN E. COLI AND SALMONELLA

Anna Knöppel, Jessica Kubicek-Sutherland

The soil and human microbiome are thought to serve as a reservoir for antibiotic resistance genes and horizontal gene transfer is a significant contributor to the spread of these genes to human pathogens. It has been demonstrated by others that exchange of antibiotic resistance genes between these nonpathogenic bacteria and clinical pathogens occurs at a relatively low frequency in natural settings. Partly this low frequency of transfer is a result of a mobilization barrier between nonpathogenic and pathogenic bacteria due to, for example, various restriction and immunity systems. However the possibility that these resistance genes are not often maintained in pathogenic isolates due to inherent fitness costs of the transferred resistance genes has not been extensively explored. In this project we are introducing a large set of different types of resistance genes isolated previously from either soil or human microbiota into neutral chromosomal positions of the Escherichia coli and S. enterica genomes under a strong inducible promoter, and we will study the stability, fitness cost and level of resistance conferred by these genes as a function of their level of expression. We also plan to evolve strains with inserts of costly resistance genes further to study the fate of the genes and the possibility for acquisition of compensatory mutations in the resistance genes themselves or elsewhere in the genomes. The primary question asked in the project is: “Could resistance genes from the environment survive in E. coli and Salmonella if transferred horizontally?”

FITNESS EFFECTS OF RANDOM MUTATIONS

Peter Lind

How and why randomly introduced mutations affect cellular function are fundamental questions in evolutionary biology and medical genetics. We utilize a bacterial model system where highly sensitive fitness assays are used to assess the function of randomly mutated gene variants in comparison with an isogenic wild type strain. Our results demonstrate that both the distribution of fitness effects and the mechanistic causes of them are dependent on which functional class the gene belongs to. Therefore we study the fitness effects of mutations in a variety of genes that encode ribosomal proteins, enzymes, transcription factors and transporters in order to get a better understanding of the fitness landscape governing evolution as well as the genetic basis for complex human diseases.

MECHANISMS OF TIGECYCLINE RESISTANCE IN ESCHERICHIA COLI

Marius Linkevicius

Tigecycline is the main representative of the new class antibiotics glycylcylines and it has been used in medical practice since 2005. It is active against multidrug resistant gram-positive bacteria like methicillin resistant S. aureus, vancomycin resistant enterococci and gram-negative pathogens producing extended spectrum ß-lactamases. However, resistance against tigecycline has been reported. Overexpression of unspecific RND or MATE family transporters was suggested as the reason for the resistance to tigecycline. This study focuses on determination of resistance mechanisms to tigecycline and the consequential fitness costs in E. coli. Two main groups of spontaneous E. coli mutants with low-level resistance to tigecycline were identified. 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 present in strains of Enterobacteriaceae, clinically resistant to tigecycline. The selected low-level tigecycline resistant mutants had increased MICs for hydrophobic antibiotics and reduced MICs for SOS inducing antibiotics. In addition, a fitness cost of these mutations was observed, as reconstructed E. coli mutants with reduced susceptibility to tigecycline grew 0.3 to 24 percent slower than the wild-type E. coli strain. A series of in vitro tests (oxidative stress, bile sensitivity, serum sensitivity and acid tolerance) was performed to further characterize reconstructed E. coli mutants. While LPS mutants demonstrated increased sensitivity to bile, none of the other in vitro tests revealed any major differences from the wild-type. Similarly, only LPS mutants were cleared out in some in vivo competition experiments. Collectively, these data indicate that the majority of parameters that can be important during infection are not affected by the mutations leading to the reduced susceptibility to tigecycline. Initial analysis of compensatory evolution results showed that it was possible to restore the growth defects caused by the resistance mutations. The compensatory mutations identified were either within the resistance gene (ERN group) or extragenic (LPS group). Further analysis should shed more light on how the compensated mutants restored the growth to the wild-type level.

TIGECYCLINE RESISTANT TET PROTEINS

Marius Linkevicius

Tigecycline overcomes major resistance mechanisms that render previous two generations of tetracyclines non-usable. It is not transported out of the cell by Tet efflux pumps, as the transport proteins cannot recognise the antibiotic as a substrate due to its bulkier chemical structure. In addition, higher affinity to ribosome and the 9-t-butylglycylamido side chain prevents the dissociation of tigecycline from the A site in the 30 S subunit even in the presence of Tet ribosomal protection proteins. In this study, we are interested 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. Selections of tigecycline resistant mutants harbouring mutagenised Tet efflux proteins revealed that it is possible to select protein variants, with increased minimal inhibitory concentration of tigecycline. Mutations leading to elevated tigecycline MIC accumulated in periplasmic loops and transmembrane domains of Tet(A) efflux protein. These mutations most probably are affecting the pore size of the pump to accommodate the bulky tigecycline molecule. All Tet(M) mutant proteins contained at least one mutation in loop III of domain IV. This loop interacts with C1054 in 16S rRNA, the nucleotide important for tetracycline binding to the ribosome and chases out tetracycline molecule from its binding site. Interestingly, amino acid substitutions or deletions present in this region increased tigecycline MIC and led to collateral sensitivity of earlier generations of tetracyclines. Further analysis and characterization of tet mutants causing reduced susceptibility to tigecycline is underway.

SALMONELLA MUTANTS RESISTANT TO ANTIMICROBIAL PEPTIDES

Hava Lofton

Antimicrobial Peptides (AMPs) are listed as promising new drug candidates that could function as a complement to antibiotics in treatment of bacterial infections. 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 determine if and how bacteria develop resistance to different classes of AMPs and the resulting effects (i.e. fitness costs) in bacteria. By daily serial passaging of small amounts of S. enterica in progressively 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). These mutations were subsequently reconstituted in a wild type genetic background. The mutations in both rfaY (phosphorylates HepII in the LPS core) and phoP (two-component global regulator) confer the major increase of the resistance, including cross-resistance, against all three AMPs. The fitness cost measured for all of the reconstituted mutants ranged from +0.8% to -16%, suggesting that AMP resistance is associated with rather small fitness effects. Importantly, pmrB and phoP mutants survived quite well in a mouse infection model with no significant difference compared to wild-type bacteria. 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 through direct resistance and/or cross-resistance to other types of human AMPs.

STAPHLYOCOCCUS AUREUS RESISTANCE TO ANTIMICROBIAL PEPTIDES

Hava Lofton, Jessica Kubicek Sutherland

S. aureus infections are among the most dangerous due to their often aggressive progression and pathogenesis as well as the ability to develop antibiotic resistance. Antimicrobial Peptides (AMPs), part of the innate immune system, are a class of molecules being developed largely for applications such as ointments for skin infections in which Staphylococcus species would be especially targeted. Since in many cases our last line of defense is our own immune system, it is therefore urgent to determine the ability of S. aureus to acquire resistance to AMPs. Daily passaging with periodically increasing concentrations of LL-37, PR-39, Wheat Germ Histones (WGHs) and a combination treatment of LL-37 and WGHs (in medium designed to mimic the mammalian ionic environment) has yielded mutants that are at the least five times that of the parent MIC. This increase in resistance to each of the AMPs, plus the combination treatment, occurred quickly (~ 200 generations.) Whole genome sequencing revealed one previously known S. aureus AMP resistance mechanism, mprF (multiple peptide resistance factor), but several others appear to be previously unknown with regards to AMP resistance. Fitness measurements show a reduction in growth rates from ~10 to 50 % that of wild-type. This implies a possible reduction in virulence, however; this remains to be tested. The rapidity of resistance emergence suggests that S. aureus can easily overcome the killing mechanisms of the AMPs used in this study.

DOES GENETIC VARIATION AFFECT EVOLVABILITY?

Erik Lundin, Joakim Näsvall

Mutations can affect the function of a gene in many different ways and may also affect later evolution of new functions by allowing the evolving enzyme to traverse fitness valleys and reach new, distant peaks in the fitness landscape. The hisA gene will be placed under the control of a strong promoter so that the activity of the HisA enzyme is not limiting for growth and mutations will be accumulated through rounds of mutagenesis. A collection of mutants containing one or several neutral mutations will be used as starting points for experimental evolution towards TrpF activity.

FUNCTIONAL TRADE-OFFS DURING EVOLUTION OF NEW FUNCTIONS

Erik Lundin, Joakim Näsvall

When a specialist enzyme (with a weak secondary activity) accumulates mutations and evolves towards a new specialist enzyme with a new function (with weak or no original activity) it will at some intermediate time points be a generalist enzyme with some original and some new activity. The mutations introduce or increase a new beneficial activity in a gene and 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. The aim of this project is to study the intermediate generalist enzymes occurring through evolution towards new gene function and determine the nature of the trade-offs when acquiring a new function and loosing an old. To test which of the trade-offs are present and assess the effects on protein stability 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 see which trade-off(s) exists in this system.

EVOLUTION OF CAPABILITIES TO UTILIZE NEW CARBON SOURCES

Erik Lundin, Ulrika Lustig, Omar Warsi, Joakim Näsvall

Environments are often fluctuating and the access to nutrients can change over time. The ability to adapt to new environments and utilize new nutrients is central to all bacteria. In this project, we aim to evolve Salmonella enterica to utilize new carbon sources. A previous study identified several hundred compounds that S. enterica cannot use as a source of carbon. Using a S. enterica strain with a high mutation frequency (due to an inducible error prone DNA-polymerase, dinB) we screened for compounds that the bacterium can use as a new carbon source. After growth of bacteria with the induced error prone DNA-polymerase in rich media, the bacteria are spread on agar plates supplemented with the compound of interest. Slow growing Salmonella enterica are collected after incubation up to 14 days on previously non-usable carbon sources. After a whole genome sequencing, point mutations have been identified in genes related to biochemical pathways involved in utilization of the new compounds.

STRATEGIES FOR ANTIBIOTIC DEVELOPMENT TO REDUCE RESISTANCE

Ulrika Lustig, Cao Sha

Antibiotic resistance in clinical settings and the decline of antibiotic drug development are increasing problems. 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 and colistin 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 models. 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 between the groups of Diarmaid Hughes and Dan Andersson (IMBIM), Lena Friberg and Mats Karlsson (FarmBio) and Otto Cars (Med Sci).

STUDY OF ANTIBIOTIC RESISTANT E.COLI IN MALLARDS

Ulrika Lustig, Marie Nykvist, Clara Atterby

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 bacteria 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) producing E. coli strains isolated from gulls to infect a set of mallards. With this in vivo model we confirmed that mallards can be infected by gull ESBL E. coli strains and the different ESBL strains were readily transmitted between birds within the group. The infection persisted in some cases for four weeks, which would allow spreading of resistant strains long distances by migrating birds. We have also studied if plasmid conjugation occurs between bacteria within the mallards and how different concentrations of antibiotic selects for antibiotic resistant bacteria in the gut of mallards. The birds were infected with an equal amount of two isogenic ESBL producing E. coli strains, one of them resistant to ciprofloxacin. During the study the mallards were exposed to concentrations ranging from 0,43-43-fold MIC of ciprofloxacin in the drinking water. In this in vivo competition we observed that ciprofloxacin resistant E. coli were selected for at a concentration of about 0,86-fold MIC in the water, probably corresponding to a much lower concentration within the bird. Transconjugant bacteria that had acquired plasmids from other strains were also detected.
This project is a collaboration between the groups of Josef Järhult, Linus Sandegren and Dan Andersson.

RESISTANCE DEVELOPMENT AND MODE OF ACTION OF CYCLOVIOLACIN O2

Sohaib Z. Malik

Cyclotides are a family of plant proteins with a cyclic backbone and three disulfide bonds that tie them into the so-called cyclic cystine knot. The extreme stability of cyclotides to chemical, thermal and enzymatic degradation makes them a promising scaffold for drug design applications. We have previously shown that the cyclotide, cycloviolacin O2 (cyO2) has a killing effect on Gram-negative bacteria in low micro-molar concentrations. In the present study, we have explored the mechanisms of resistance development to cyO2. For this purpose, 14 independent lineages of Salmonella enterica and 4 independent lineages of Escherichia coli were serially passaged in increasing concentrations of cyO2 for 100 or 150 cycles (600-700 or 900-1050 generations) to select for mutants with reduced cyclotide susceptibility. Clones were isolated from the populations evolving under this selective pressure and whole genome sequenced. Whole genome sequencing identified a number of mutations that conferred

resistance. Mutations that appeared in more than one resistant clone were reconstituted in a wild type background. All but one of these mutations reduced susceptibility to cycloviolacin O2. Cross-resistance to other antimicrobial peptides was tested. Growth rates of these mutants, relative to congenic wild type strains, were determined. As the next step, the effects of combinations of resistance mutations will be tested. In another part of this project: a) interaction of cyO2 with the outer membrane of Gram-negative bacteria was investigated using hydrophobic probes and organisms with a tighter permeability barrier, b) the effect of different culture conditions (bacterial growth phase, effect of bacteriostatic drugs, pH, inoculum size) was explored, c) antibodies were raised against cycloviolacin O2, which will be used in quantification of cyclotide binding to bacterial cells. This project is a collaboration with Prof. Ulf Göransson at Uppsala University.

ANTIMICROBIAL EFFECTS OF CYCLOTIDES

Sohaib Z. Malik

Cyclotides possess many positive attributes from a drug development perspective. Extreme stability to thermal, chemical and biological degradation, together with potent activity of the prototype bracelet cyclotide, cycloviolacin O2, against Gram-negative bacteria; warrants an in-depth exploration of their potential as a family of antimicrobial peptides. We isolated sixteen (16) cyclotides from three different plants; Oldenlandia affinis, Viola odorata and Viola arvensis. An initial screen in E. coli, revealed two cyclotides with comparable inhibitory activity to cycloviolacin O2. Another three peptides, were moderately active while the rest were inactive. NMR was done on one of the most active and one moderately active peptide. Cross-resistance to cycloviolacin O2 resistant mutants was tested. We will broaden the screen, by adding more cyclotides (natural and synthetic) and by testing against a broader set of bacteria. These studies would be a stepping-stone for the attempts to engineer cyclotides as antimicrobial therapeutics. This project is a collaboration with Prof. Ulf Göransson at Uppsala University.

PASSIVE ANTIBIOTIC RESISTANCE

Hervé Nicoloff

Passive resistance describes the protective effect that an antibiotic-resistant population can exert on a sensitive population. It is observed during coinfections involving both β-lactam-resistant and β-lactam-sensitive bacterial populations. During these, β-lactamases in resistant cells efficiently decrease the antibiotic concentration in the cells surrounding environment over time, allowing nearby β-lactam-sensitive bacteria to resume growth. The unique localization of β-lactamases in the periplasm combined with the efficient water-dependent drug-inactivation mechanism led to the speculation that passive resistance could be specific to β-lactams and β-lactamases. In this project, we investigated whether passive resistance could be detected in other situations. We tested passive resistance to thirteen antibiotics from seven different classes using fifteen resistance markers. For each antibiotic, growth of a sensitive population mixed with a resistant population carrying a plasmid-encoded resistance marker was monitored at different drug concentrations. Passive resistance was observed when the sensitive population could grow at a drug concentration higher than its minimal inhibitory concentration measured when mixed with another sensitive population (in a control

experiment). We observed passive resistance to several antibiotics and with antibiotic-modifying enzymes active in the cytoplasm and requiring cofactors such as acetyl-CoA. Thus, the unique characteristics of β-lactamases were not required for passive resistance to those drugs. Rather, we found that passive resistance was dependent on the drug diffusion rate through the cell membranes. While the concentration of efficiently-diffusing antibiotics, which require efficient drug-modifying enzymes for resistance, could decrease over time in presence of a resistant strain and lead to a passive resistance phenotype, slow-diffusing drugs could not. This work revealed that co-infections might be a greater cause of antibiotic treatment failures than previously thought.

HETERORESISTANCE

Hervé Nicoloff, Karin Hjort

Heteroresistance is the ability of bacterial isolates to grow as mixed populations characterized by different antibiotic resistance phenotypes. While the main population remains relatively sensitive to an antibiotic, a small unstable subpopulation presents a higher resistance. Heteroresistance has been described in clinical settings for a few bacterial species and antibiotics and is a great cause of concern during antibiotherapy. It is difficult to detect and its mechanism remains unknown or unclear. In this project, we wanted to assess the frequency of heteroresistance and describe the mechanisms leading to heteroresistance. To assess heteroresistance frequency, fourty clinical isolates from four different species (E. coli, S. enterica, K. pneumoniae and A. baumannii) were tested for heteroresistance against more than twenty antibiotics, most of which are of clinical interest. All strains tested presented a heteroresistance phenotype to at least one antibiotic, and heteroresistance was found for most antibiotics. The instability and the frequency of the resistant subpopulations observed (typically around $10^{-4}/10^{-5}$) could be compatible with genetic duplications/amplifications events. Therefore, to find the mechanisms of heteroresistance, subpopulations with increased resistance were selected, tested for instability of the resistance, and sent for whole genome sequencing. Potential mutations involved in heteroresistance will be detected by comparing the sequences of isolates under antibiotic selection to sequences of the same isolates grown without antibiotic selection. Eight isolates from three different species and with unstable heteroresistance phenotypes to five antibiotics were sequenced so far. This project revealed high frequencies of heteroresistance phenotypes and ongoing work will hopefully lead to the description of heteroresistance mechanisms.

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. This model system (evolution of TrpF activity in HisA) will be used to study several different factors influencing the evolution of new genes (see Erik Lundin's projects). A follow-up to this study will determine the structure, stability, expression and specific activities of the evolved enzymes (See Annika Söderholm's project).

EVOLUTION OF BACTERIAL CLASS I PEPTIDE RELEASE FACTORS

Joakim Näsvall

Protein synthesis on the ribosome is terminated by class I peptide release factors, which specifically recognize stop codons in the ribosomal A-site and trigger hydrolysis of the peptidyl-tRNA. Eukaryotes and archaea have only one class I release factor (eRF1 and aRF1, respectively) that recognize all three stop codons, while most bacteria have two release factors (RF1 and RF2) that only recognizes two stop codons each. RF1 (encoded by the prfA gene) recognizes UAA and UAG, RF2 (encoded by the prfB gene) recognizes UAA and UGA. It has previously been assumed that both of the bacterial Class I release factors are essential, but recent studies have demonstrated that RF1 is not essential in Escherichia coli if RF2 is fully functional. Experimental adaptive evolution of a bacterium lacking one of the release factors can be used in the context of studying evolution of new genes, e.g. evolution of a "new RF1" from a duplicate of the prfB gene or evolution of an eRF1/aRF1-like release factor that recognizes all three stop codons efficiently. It can also be used to study evolution of the genetic code itself, e.g. codon reassignments, and give clues to why almost all bacteria have two class I release factors when archaea and eukaryotes suffice with only one, omnipotent, release factor. We have constructed a Salmonella enterica strain lacking the prfA gene. In Salmonella the prfA gene appears to be essential, unless the strain also has an amplification of the prfB gene. Such a strain is extremely slow growing, leaving plenty of room for adaptive evolution by selection for faster growth. We have allowed this mutant to undergo adaptive evolution by serial passage of multiple populations. In only a few tens of generations of growth under selection, all 16 populations increased their growth rates by amplifying the prfB gene to high copy numbers. During 500 generations of adaptive evolution, several populations acquired additional mutations in prfB. Mutations in other components of the translational machinery, including Release Factor 3, ribosomal protein S9, tmRNA and three 16S rRNA modification enzymes accumulated in several populations. Currently we are re-constructing strains with these candidate adaptive mutations to determine which mutations compensate for the loss of RF1. It is notable that the deleterious effects caused by lack of a release factor can be compensated by such an extensive variety of mutations involving protein synthesis.

INSIGHTS INTO ENZYME EVOLUTION BY CRYSTALLOGRAPHIC STRUCTURE DETERMINATION OF HISA MUTANTS POSSESSING TRPF ACTIVITY

Annika Söderholm

In the study ”Real-time evolution of new genes by innovation, amplification, and divergence” by Näsvall et al. 2012 it was demonstrated how new genes can evolve by the innovation-amplification and divergence evolutionary (IAD) model. In the study, a mutated hisA gene from S. enterica which provided a low level of TrpF activity while retaining some original activity was isolated and placed in a strain that lack functional hisA- and trpF genes. Through continuous selection for both activities in the absence of histidine and tryptophan amplification of the dual function hisA gene was promoted and the amplified gene products diverged so that different mutant enzymes evolved. The evolved mutants could be divided into three different categories related to their catalytic characteristics; HisA specialists, TrpF specialists or generalists. The aim of this project is to provide a structural explanation of how the different mutations affect the two catalytic activities and to understand what is the basis for generalist and specialist activities. An additional aim of the project, that arose due to discrepancies in previously published literature regarding the catalytic mechanism of HisA, is to establish the correct mechanism of this enzyme. I work together with Xiaohu Guo (Maria Selmer’s group, ICM) on determining the structures of the wild type and mutant HisA enzymes by X-ray crystallography. Furthermore, the stability of the proteins is being examined using circular dichroism spectroscopy. Our structure and stability data is analyzed together with enzyme kinetic data provided by Wayne Patrick’s group (University of Otago, New Zealand). We have solved structures of 11 different S. enterica HisA mutants as well as the wild type enzyme. We also have several ligand structures both with the HisA substrate ProFAR and with the TrpF product analogue rCdRP. Based on the results we can now dismiss the previously suggested catalytic mechanism for HisA and provide new insight into its mode of catalysis. We can also provide hypotheses on how HisA evolved TrpF activity and suggest what the roles are of the different mutations that were acquired in the evolution study. Although some of the hypotheses are still being tested we are in the final phase of this project. We are currently working on a manuscript concerning the results providing mechanistic insight of the wild type HisA enzyme and how the isomerization reaction occurs. A second manuscript will concern the evolutionary aspects of adaptation and how the kinetic parameters of the enzymes is correlated to the growth phenotypes of the evolved mutant genes.

MECILLINAM RESISTANCE

Elisabeth Thulin

Many of the traditional antibiotics used for treatment of urinary tract infections (UTIs) have been rendered useless due to the resistance development in the UTI-causing pathogens. But resistance development to mecillinam has remained low and it is now used more widely for treatment. The goal of this project is to understand the dynamics of resistance development, ultimately with the aim to minimize it. 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 laboratory. Characterization of the mutants and identification as well as reconstruction of the resistance mutations has been done for both species. We found several novel mecillinam resistance genes. The mecillinam resistant clinical isolates were whole genome sequenced and compared to 20 reference strains (both clinical and isolated in the lab). The clinical isolates have significantly higher fitness compared to the in vitro mutants both in media with and without mecillinam added. Mutations in one particular mecillinam resistance gene (cysB) appeared in all the clinical strains. These mutations confer resistance above the clinical break point for mecillinam, but cannot alone explain the very high resistance shown in a few of the clinical isolates. The clinical isolates might have this mutation because it has a low fitness cost, and since it gives an intermediate resistance it might be important as a stepping stone for development of higher resistance in clinical settings. Several other mutations that might contribute to higher resistance were also found in the clinical isolates. Presently we are comparing growth of in vitro and clinical mutants in urine (clinical isolates are from UTI patients), examining several different types of epistasis in mecillinam resistance and working on getting a better understanding of the impact of ppGpp (stringent response) on mecillinam resistance.

FITNESS COMPENSATION OF MECILLINAM RESISTANT MUTANTS

Elisabeth Thulin, Michael Knopp

In a serial passage evolution experiment mecillinam resistant mutants (obtained from the previously described project) that had compensated for the fitness loss (decreased growth rate) associated with mecillinam resistance were selected and sequenced to identify the mutations responsible for the growth compensation. Reconstruction of the compensatory mutations to determine their effects on fitness and resistance is ongoing. In addition, we have found mecillinam resistant clinical isolates that appear to have acquired compensatory mutations. These have been whole genome sequenced and we are comparing them to clinical isolates from the same patient as well as to the laboratory selected compensated strains to identify the compensatory mutations and whether they are the same in clinical isolates and laboratory selected mutants.

BACTERIAL RESPONSES TO STRESS AND SELECTION

Diarmaid Hughes

Our research interests include 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. We also lead the microbiology effort in the IMI ND4BB ENABLE project, a public-private partnership involving over 30 different groups in Europe (large pharmaceutical companies, small to medium enterprises, hospitals, research institutes, and universities), with the aim of developing novel antibiotics active against Gram-negative organisms and taking at least one into phase 1 clinical trials.

We are studying the development of resistance to antimicrobial drugs, with a particular focus on the fluoroquinolones and rifampicin. 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.

Within ENABLE we are involved in testing the antibacterial activities of novel compounds targeting Gram-negatives, and with testing resistance frequencies and resistance mechanisms, employing whole genome sequencing and genetics.

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.

Members of the group during 2014
Diarmaid Hughes, Professor
Douglas Huseby, Researcher
Cao Sha, Researcher
Lisa Praski Alzrigat, PhD student
Jessica Bergman, PhD student
Gerrit Brandis, PhD student
Eva Garmendia, PhD student
Linnéa Garoff, PhD student
Franziska Pietsch, PhD student

Project workers during 2014

Linnéa Garoff (Masters project, Spring term)
Andreas Lipfert (Masters project, Spring term)
Rongpong Plongla (Masters Project, Spring term)
Punya Pallabi Mishra (Research project, Autumn term)
Angelica Tegehall, Masters project, begun Autumn term)

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EXTRAGENIC SUPPRESSORS OF RNase E MUTANTS

Disa Hammarlöf, Jessica Bergman, Eva Garmendia

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.

REGULATION OF THE *tufB* OPERON IN *SALMONELLA*

Gerrit Brandis, Jessica Bergman

In *Salmonella enterica* and related species, translation elongation factor EF-Tu is encoded by two widely separated but near-identical genes, *tufA* and *tufB*. Two thirds of EF-Tu is expressed from *tufA* with the remaining one third coming from *tufB*. Inactivation of *tufB* decreases total EF-Tu in the cell by one third. In contrast, inactivation of *tufA* is partly compensated by a doubling in the amount of EF-TuB. How the cell senses a shortfall in EF-Tu, and how it regulates a response by increasing expression from *tufB* is unknown. We are using genetics to address the mechanism by which *tufB* expression is regulated in *Salmonella*. By experimental evolution of a strain with an inactive *tufA* gene we selected three different non-coding or synonymous point mutations close to the *tufB* start codon. Using computational methods, we identified a conserved sequence in the region just upstream of, and within the early region of the *tufB* coding sequence. This conserved sequence could be computationally folded into a structure that potentially occludes the *tufB* ribosome binding sequence. We have created a series of mutations throughout this sequence to test whether the putative structural features were significant for regulating *tufB* expression.

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. The 64 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 physiological consequences of changing codon usage will be assessed to measure the selective value of biased codon usage. The aim is to understand the physiological significance of codon usage bias in highly expressed genes and how rapidly codon usage bias could evolve under selection.

REDUCING 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. It was found that during serial passage of *Escherichia coli* strains carrying classical drug-resistance plasmids, that the costs of carriage were rapidly reduced; furthermore, it was shown that the cost reduction 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.

LOCATION AND ORIENTATION OF CRITICAL 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 DNA is replicated. The current hypothesis is that genomic-scale organization reflects selection pressure for maximum growth rate, by increasing the copy number of highly transcribed genes and minimizing the frequency of clashes between DNA and RNA polymerases. This study is testing the significance of position and orientation of *tuf*, a gene whose product, EF-

Tu, is directly liked to growth rate. EF-Tu is the single most abundant cytoplasmic protein in both *Escherichia coli* and *Salmonella typhimurium*. The *tuf* gene 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 position and orientation. Evolution experiments will address whether sub-optimal location and/or orientation can be compensated and by which mechanisms.

RNA POLYMERASE MUTATIONS ARE SELECTED DURING EVOLUTION OF CIPROFLOXACIN RESISTANCE IN *E. COLI*

Franziska Pietsch

E. coli is naturally sensitive to the DNA gyrase inhibitor ciprofloxacin. Resistance requires multiple mutations or other genetic alterations. Independent lineages of *E. coli* were cycled in the presence of ciprofloxacin, at progressively higher concentrations of drug, and end-point strains were then analyzed by whole genome sequencing. In addition to mutations in known resistance-associated genes, we also found mutations in *rpoB* and *rpoC*, coding for the β– and β'-subunits of RNA polymerase. This suggests that mutations affecting RNA polymerase arise commonly under these selective conditions. Strains representing the successive steps in the resistance evolution were constructed and characterized for susceptibility to ciprofloxacin and their impact on the bacterial fitness. In order to better quantify the selective advantage conferred by the identified mutations, competition experiments between successive pairs of constructed strains were performed at different ciprofloxacin concentrations. Each of the successive mutations in each lineage provided a competitive advantage relative to the previous genotype, as a function of increasing drug concentration. In addition, each of the selected polymerase mutations confers a significant increase in ciprofloxacin resistance in isogenic wild-type background strains as well as in different ciprofloxacin resistant background strains selected during the evolution.

SIGNIFICANCE OF DIFFERENT ALLELES OF *gyrA* SELECTED DURING THE EVOLUTION OF RESISTANCE TO CIPROFLOXACIN

Franziska Pietsch

Fluoroquinolones, such as ciprofloxacin, target the essential enzymes DNA gyrase and DNA topoisomerase IV, thereby inhibiting bacterial chromosome replication and transcription. Mutations in *gyrA* typically appear early in the development of ciprofloxacin resistance evolution and are present in all resistant *E. coli* strains. Although many different *gyrA* mutations can lead to an increased ciprofloxacin resistance, a bias towards a small set of mutants has been observed. This project aims to understand why particular *gyrA* alleles dominate, both in evolved laboratory strains and in clinical isolates. The hypothesis that particular alleles of *gyrA* confer a selective advantage is being tested by constructing isogenic strains, carrying mutant *gyrA* alleles frequently identified in evolution experiments or commonly found in clinical strains. Strains will be characterized with respect to drug resistance and growth rate. Competition experiments will be performed at different drug concentrations and the influence of the mutant alleles on supercoiling and transcription rate

will be determined. The possibility that different *gyrA* alleles differentially predispose to subsequent success in evolving to higher levels of resistance will be tested in laboratory evolution experiments, coupled with analysis of the outcome by whole genome sequencing.

SPONTANEOUS FRAMESHIFT MUTATION SUPPRESSION

Douglas Huseby, Lisa Praski Alzrigat, Gerrit Brandis

Frameshift mutations alter the translational reading frame in coding sequences, and are not unusual mutational events. Frameshifts are practically always inactivating mutations, since all codons downstream of the mutation will be incorrect. During an experimental evolution, we isolated an unusual frameshift mutation in an essential gene in *Escherichia coli*. Defying our expectations, a strain carrying this mutation is able to survive without any additional frameshift-suppressing mutations. Our investigations have determined that the sequence surrounding the site of the mutation spontaneously suppresses the frameshift, allowing the ribosome to slip back into the correct reading frame with extraordinarily high frequency. Our model for this suppression, for which we have accumulated considerable evidence, invokes a hungry codon preceded by a codon that is prone to slipping, both of which must be present in a gene that is highly expressed. This mechanism of frameshift suppression has been observed before, but only in artificial constructions. Subsequent experimental evolutions have identified other mutations that disrupt the reading frame of different essential genes. This suggests to us the tantalizing possibility that spontaneous suppression of frameshift mutations may not be as unusual as believed.

FITNESS COSTS AND COMPENSATION IN FLUOROQUINOLONE RESISTANCE DEVELOPMENT

Lisa Praski Alzrigat, Douglas Huseby

Resistance to fluoroquinolones can arise through mutations that affect the drug targets; by mutations that inactivate repressors of the AcrAB-TolC multi-drug efflux system; and by acquisition of genes conferring a protective effect on the antibiotic targets. Resistance-conferring genetic alterations could a fitness cost to strains carrying them. We have experimentally evolved lineages of *Escherichia coli* that are highly resistant to ciprofloxacin, by subjecting strains to increasing concentrations of the antibiotic over hundreds of generations of growth. The antibiotic resistant mutants have been subjected to whole-genome sequencing. In addition to mutations known to confer resistance, a variety of non-canonical mutations were also observed in these strains. During the process of these laboratory evolution experiments, it is expected that there will be selection for resistance, and also mutations that reduce the fitness cost of resistance mutations. We are analyzing the contribution of non-canonical mutations to the final phenotype to determine whether they contribute directly to resistance or whether they mitigate the fitness costs of resistance mutations. This analysis will contribute to a fuller understanding of resistance evolution.

MUTATIONAL BIAS IN EFFLUX MUTANTS

Lisa Praski Alzrigat

AcrAB-TolC is the major efflux pump associated with resistance to ciprofloxacin in *E. coli*. Mutations that lead to overexpression of AcrAB-TolC increase the MIC for ciprofloxacin. Expression of AcrAB-TolC is regulated by the local repressor *acrR* (*acrA* and *acrB*) and the global transcriptional regulator *marA*, which in turn is regulated by the repressor *marR*. Knockout mutations in either *acrR* or *marR* cause increased expression of the AcrAB-TolC efflux pump. Because neither *marR* nor *acrR* is essential, knock out mutations in these genes are expected to arise at a higher frequency than specific single amino acid substitutions. However, a surprisingly high frequency of amino acid substitutions is found in these regulator genes in resistant isolates. We are assaying the phenotypes of individual *acrR* and *marR* mutations observed in clinical isolates by creating isogenic mutant strains and measuring MICs and fitness costs. This is to test the hypothesis that knockout mutations are relatively costly and that the specific mutations selected in clinical isolates successfully balance fitness costs with the advantage of increased resistance.

FUNCTIONAL SIGNIFICANCE OF tRNA SYNTHETASE MUTATIONS IN EVOLUTION OF RESISTANCE TO CIPROFLOXACIN IN *E. COLI*

Linnéa Garoff, Douglas Huseby

Ciprofloxacin is a commonly used fluoroquinolone that targets the essential enzymes DNA gyrase and topoisomerase IV. Resistance to fluoroquinolones in *E. coli* is known to arise by mutations altering the target protein genes and by mutations increasing drug efflux, however resistance above the clinical breakpoint requires multiple mutations. In an *in vitro* evolution experiment selecting for increasing resistance to ciprofloxacin, mutations in tRNA synthetases arose frequently. Isogenic *E. coli* strains were created and tested for phenotypes of these mutations affecting resistance or bacterial fitness. Mutations in *leuS* increased MIC and reduced growth rate when in combination with a known resistance mutation. When grown in the presence of ciprofloxacin, strains with a *leuS* mutation in combination with a resistance mutation had a significant growth advantage that was dependent on their ability to activate the stringent response. Taken together, our findings suggest that the *leuS* mutations may have been selected as compensatory mutations that increase the fitness of *E. coli* strains carrying primary resistance mutations, and that the increased fitness is due to an induction of the stringent response via activation of RelA.

IDENTIFICATION OF SUPPRESSORS OF THE SCV PHENOTYPE IN STAPHYLOCOCCUS AUREUS

Cao Sha, Douglas Huseby

Small colony variants (SCVs) of *S. aureus* are associated with reduced susceptibility to aminoglycosides and enhanced persistence in mammalian cells. Most SCVs have mutations in hemin or menadione biosynthesis genes. When SCVs were selected for faster growth, they acquired, in most but not all cases, compensatory mutations in the *hem* or *men* biosynthetic genes. The aim of this project is to identify the non-canonical mutations (outside of the

primary *hem* and *men* sites) that reversed the SCV phenotype. We used whole genome sequencing to analyse SCVs and growth-compensated mutants. In addition to growth compensation by mutations in hemin and menadione biosynthesis genes, we identified mutations in tRNA genes that improve growth rate by translational suppression of the original SCV mutant alleles. DNA sequencing of an additional class of suppressors is in progress to determine whether an alternative bypass mechanism exists that can suppress the SCV phenotype. We have tentative evidence for the involvement of genes involved in regulating the accumulation of osmoprotectants. The addition of osmoprotectants to the growth medium also significantly improved the growth rate of several of the SCVs.

ENABLE GRAM-NEGATIVE DRUG DISCOVERY PLATFORM

Cao Sha, Karin Hjort, Doug Huseby

As part of the Uppsala University-led ENABLE consortium we are carrying out *in vitro* microbiology assays on novel drug hits and leads, including MIC assays involving a hierarchy of different strain panels, hemolysis assays, time-to-kill assays, measurements of the frequency of resistance, resistance-fitness assays, and whole genome sequencing together with genetic reconstruction to identify resistance mechanisms. The project is set up to efficiently process a pipeline of novel compounds with the ultimate aim of identifying one or more candidate drugs for continuation into Phase 1 clinical trials.

DYNAMICS 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. 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, how do they affect the efficacy of antibiotics and does the dynamics of gene amplification on plasmids accelerate evolution of new resistance?

4. Can targeting of resistance plasmids be a way to eliminate resistance genes 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 accelerate 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.

Members of the group during 2014:
Linus Sandegren – Associate professor
Marlen Adler – Postdoc
Fredrika Rajer – PhD student
Erik Gullberg – PhD student (see also Dan Andersson)
Marius Linkevicius – PhD student (see also Dan Andersson)

Project students during 2014:
Fredrika Rajer
Cecilia Strömhielm

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ESBL-PLASMID 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 how the outbreak clone and the resistance plasmid have changed 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 another plasmid have occurred through direct homologous recombination, in part mediated through homology in shared mobile insertion sequences between the two plasmids. 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 have now also completed the genome sequence of the outbreak clone and are performing comparative analysis of 110 isolates of the outbreak clone looking into how both the plasmid and the chromosomal sequence has changed during the outbreak.

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

Fredrika Rajer, Erik Gullberg

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 properties of plasmid fitness costs and how they can be compensated for. We also study segregational stability properties of plasmids in different genetic backgrounds to understand why some plasmids are very stably inherited in one 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.

CARBAPENEM RESISTANCE IN E. COLI

Marlen Adler

The use of last resort antibiotics such as carbapenems has increased in response to the worldwide spread of extended-spectrum β-lactamase (ESBL) producing pathogens. Within several projects we are studying the mechanisms by which bacteria can spontaneously 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. Our studies show that the spectra of mutations and the final resistance levels (minimal inhibitory concentration MIC) differ between carbapenems. Furthermore, the production of β-lactamases increases the carbapenem MICs and allows for a wider spectrum of mutations.

To study the effects of less frequent mutations on the development of carbapenem resistance we conducted a study with mutants that already lacked OmpC and OmpF and serially passaged them at increasing carbapenem concentrations. All resulting mutations affected the AcrAB-TolC efflux system and targeted the penicillin-binding protein (PBP) specific for each carbapenem. Ertapenem resistance mutations targeted PBP3 while meropenem resistance mutations targeted PBP2 or induced the stringent response rendering PBP2 non-essential.

EVOLUTION OF CARBAPENEM RESISTANCE IN KLEBSIELLA PNEUMONIAE

Marlen Adler, Cecilia Strömhielm

We are interested in the detailed genetic mechanisms by which *Klebsiella pneumoniae* can develop resistance to carbapenems. Previous reports show that the mechanisms employed by *K. pneumoniae* and *E. coli* are similar, but porin deficient ESBL-producing *K. pneumoniae* are much more common in clinical settings than their *E. coli* counterparts. We are especially interested in the differences that allow *K. pneumoniae*'s more frequent associations with nosocomial outbreaks.

The outbreak of ESBL-producing *K. pneumoniae* at the Uppsala University Hospital gives us the opportunity to study the evolution and spread of carbapenem-resistance in over 100 clinical isolates over a time of five years. We also performed step-wise selections for increased carbapenem-resistance with sensitive reference strains and clinical outbreak strains. Characterisation of these mutants showed rapid and strong resistance development for the outbreak strains, whereas the reference strain needed additional selective steps to reach comparable resistance levels.

Just as with *E. coli* decreased expression of porins serves as the first line of defence but for *K. pneumoniae*, loss of one porin alone is sufficient for significantly lowered drug susceptibility.

The sensitive strain did not employ any of the previously seen mutations to decrease its susceptibility and whole genome sequencing will be necessary to discover the exact mutations involved.

Initially we hypothesised that the reason for the more frequent *Klebsiella* infections would be a lower biological cost for the same mutations as compared to *E. coli*. This proved not to be the case and more experiments will be needed to learn why carbapenem resistance develops more frequently in *K. pneumoniae*.

INSTABILITY AND COST OF GENE AMPLIFICATION REDUCE RATES OF EVOLUTION OF NEW GENES

Marlen Adler

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. These amplifications also increase the probability to accumulate mutations that would allow better catalytic activity towards carbapenems, the evolution of a new gene.

A major mechanism for generation of new genes is by duplication-divergence. Here an existing gene is first duplicated and later diverges through accumulation of neutral mutations into different genes. Duplication-divergence includes several different sub-models: i) subfunctionalization where the original function is distributed between two partially functional and complementary genes and ii) neofunctionalization where a new function evolves in one of the duplicated copies while the old function is maintained in another copy. The likelihood of these mechanisms depends on the stability of the duplicated state, which in turn depends on the fitness cost and genetic stability of the duplications.

We experimentally determined the fitness cost and stability of defined gene amplifications of beta-lactamase genes on our ESBL-plasmid. Our results show that the costs of carrying extra gene copies are substantial and that each additional kbp of DNA reduces fitness by approximately 0.15%. Furthermore, gene amplifications are highly unstable and rapidly segregate to lower copy numbers in absence of selection. Mathematical modelling shows that the fitness costs and instability strongly reduces the likelihood of both sub- and neofunctionalization, but that these effects can be off-set by positive selection for novel beneficial functions such as resistance in the presence of antibiotics.

EVOLUTION OF TIGECYCLINE RESISTANCE

Marius Linkevicius

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 in addition to the overexpression of AcrAB efflux system, spontaneous *E. coli* mutations in LPS biosynthesis also lead to reduced susceptibility to tigecycline. Both groups of mutations (efflux and LPS) come with a fitness cost and while efflux mutants behave similarly to wild-type in the mice model systems tested, the LPS mutants are cleared out from some *in vivo* infection models, suggesting difficulties establishing themselves within the host. Additionally, the *in vitro* compensatory evolution experiments show that the observed fitness cost can be alleviated by acquiring mutations within the resistance gene (efflux) or in other targets (LPS).

Horizontally disseminated *tet* resistance determinants can also evolve to cause reduced susceptibility to tigecycline. We have found accumulation of such mutations in Tet(A) pump and Tet(M) ribosomal protection protein. While the Tet(A) mutations most probably lead to channel enlargement to fit a bulky tigecycline structure, the Tet(M) mutations affect the loop interacting with the tigecycline binding-site within the ribosome. The spread of such mutant variants might have negative implications for the use of tigecycline in the future.

SYNTHETIC CRISPR SYSTEMS TARGETING RESISTANCE GENES

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 comprises 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 MALLARDS (ANAS PLATYRHYNCHOS)

Ulrika Lustig, Marie Nykvist, Clara Atterby

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 bacteria 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) producing *E. coli* strains isolated from gulls to infect a set of mallards. With this *in vivo* model we confirmed that mallards can be infected by gull ESBL *E. coli* strains and the different ESBL strains were readily transmitted between birds within the group. The infection persisted in some cases for four weeks, which would allow spreading of resistant strains long distances by migrating birds.

We have also studied if plasmid conjugation occurs between bacteria within the mallards and how different concentrations of antibiotic selects for antibiotic resistant bacteria in the gut of mallards. The birds were infected with an equal amount of two isogenic ESBL producing *E. coli* strains, one of them resistant to ciprofloxacin. During the study the mallards were exposed to concentrations ranging from 0-43-fold MIC of ciprofloxacin in the drinking water. In this *in vivo* competition we observed that ciprofloxacin resistant *E. coli* were selected for at a concentration of about 0,86-fold MIC in the water, corresponding to a much lower concentration within the bird. Transconjugant bacteria that had acquired plasmids from other strains were also detected.
This project is a collaboration between the groups of Josef Järhult, 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. In malaria treatment, sulfonamides are still 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 2014
Göte Swedberg, associate professor
Nizar Enweji, PhD student, graduated November 12, 2014
Catherine Lwanira, PhD student (Makerere University, Kampala, Uganda)
Lemu Golassa, PhD student (Addis Ababa University, Ethiopia)
Carol Marwa, PhD student (Catholic University of Health and Allied Sciences, Mwanza, Tanzania)
Boniphace Sylvester, PhD student (Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania)

Project workers during 2014
Özlem Koca: Betydelsen av ICE och MEGA för resistensegenskaper hos *S. parasanguinis* och *S. salivarius.*
Sama Latif: Undersökning av HPPK-DHPS och dess funktion efter specifika deletioner.
Farah Said: Detection of resistance markers for antifolates in *Plasmodium vivax*
Zahra Mahdi: Screeningsstudie om förekomst av resistensmarkörer för klorokin i Etiopien
Marwa Mami: Mutationer relaterade till resistens mot malarialäkemedlet artemisinin
Maria Tusell Rabassa: Variation in Pfmdr1 in *Plasmodium falciparum* from Tanzania and Ethiopia
jeThi Cam Linh Nguyen: Exploring the genetic diversity of the malaria parasite *Plasmodium vivax* in Ethiopia.
Hadeel Ali: Emerging resistance to artemisinin; are polymorphisms in the gene *pfubp1* linked to artemisinin resistance?
Meha Saad Jaber: Emerging resistance to artemisinin; are polymorphisms in the gene *pfap2mu* linked to artemisinin resistance?
Hussein Abdulwahab: Förekomsten av genetisk variation i och omkring *pfcrt*-genen hos *Plasmodium falciparum* från Etiopien.
Sabri Kardi: Variation hos *pfmdr1* och *pfcrt* i isolat från *Plasmodium falciparum* positiva prover från Etiopien och Tanzania.
Kwang Torng Sim: Biodegradation of Sulphonamides and Ciprofloxacin in Pseudomonas putida and the Involvement of Plasmids.

International exchange during 2014

Lemu Golassa, Addis Ababa University, Ethiopia, worked in the lab March to June.
Boniphace Sylvester, Tanzania, worked in the lab August to October.
Göte Swedberg visited partners in Tanzania in February.

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Agencies that support the work

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RESISTANCE TO ANTIMALARIAL DRUGS AND EVALUATION OF NEW DRUG TARGETS

Nizar Enweji, Lemu Golassa

Several antimalarial drugs act on the folate metabolism affecting synthesis of DNA precursors, especially dTTP. 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 antimalarial 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 Uganda, Tanzania and Ethiopia. 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 result from this change in drug use. No signs of artemisinin resistance were detected. In Ethiopia malaria due to *Plasmodium vivax* is common, and the first choice for treatment is chloroquine. This may explain our findings that one main marker for chloroquine resistance in *Plasmodium falciparum* has a low frequency in Tanzania while the frequency is still high in Ethiopia. In Ethiopia we could also detect a large number of asymptomatic carriers for both parasites, a finding that has implications for control of malaria. The parasite population in Tanzania shows much greater variation than in Ethiopia by a number of indicators, including microsatellite markers, as well as variations in potential drug resistance genes.

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.

HOST FACTORS INVOLVED IN SUSCEPTIBILITY TO MALARIA INFECTIONS

Catherine Lwanira, Boniphace Sylvester, Carol Marwa

An infection is an interplay between parasite and host. While most of our previous work has been focused on the parasite, some new projects focus on host factors. These include genetic polymorphisms in blood groups, other blood related variations and immune system factors. These factors are investigated by Catherine Lwanira with the purpose of linking host polymorphisms to susceptibility to infection in a cohort of children living in a village in Eastern Uganda, where malaria is still very prevalent.

Boniphace Sylvester focus his work on malaria during pregnancy, and the impact on the mothers immune status on the development of immunity and susceptibility to malarial infections in newborn children. So far, only mothers with acute malarial infections has been studied, but a more thorough investigation of the role of asymptomatic infections in pregnant women is planned.

Still another host factor of interest is polymorphisms in cytochrome P450 enzymes involved in drug metabolism. There are substantial genetic differences between people from different countries. Since the arsenal of antimalarial drugs is limited, the same or very similar drugs are used in all malaria afflicted areas, but drug metabolism can be quite different in Africa as compared to South East Asia and South America. Therefore, aspects of drug metabolism are important for choice of drugs and the dosing during treatment. A complicating factor is interactions between antimalarials and antiretroviral drugs. Especially in Tanzania there is a substantial overlap in HIV and malaria infections, where this issue is important. The PhD project of Carol Marwa, Mwanza, Tanzania will address these issues.

MOLECULAR VIROLOGY AND VIRAL ZOONOSES

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

We are using viruses as model systems to study gene expression both in normal cells and under stress conditions and disease. Viral reprogramming of 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 human health and regulatory networks at the cellular and organism level we are using human 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. 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 develop novel strategies to use viruses in medical applications.

Most human infections are zoonotic, which means that they can cross species barriers and pass from animals to humans, or vice versa. In a second line of research we are focusing on zoonotic viruses where we use an interdisciplinary approach based on molecular virology, immunology, genetics, molecular epidemiology and virus diagnostics to study viral zoonoses. The present work is focused on several medically important virus families like hantaviruses, flaviviruses, Sinbis virus, Rift Valley fever virus and avian influenza virus.

ADENOVIRUS IN BASIC AND MEDICAL RESEARCH

Göran Akusjärvi, 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, viruses typically need to manipulate crucial regulatory nodes in cells to reprogram them into virus-producing factories. In fact, 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 virus gene expression with an ultimate goal to use our gained knowledge for design of safer viral vectors for medical applications.

We study:

- Regulation of RNA splicing and transcription by the viral L4-22K and L4-33K proteins
- The structure and function of adenoviral miRNAs
- Long-term persistent/latent adenovirus infections in human tonsils
- Adenovirus control of non-coding RNA expression and cholesterol metabolism
- Novel functions of the adenoviral E1B oncoproteins
- Viral vectors in cancer therapy

Members of the groups during 2014

Göran Akusjärvi, professor, group leader
Göran Magnusson, senior professor
Daniel Öberg, researcher, group leader
Farzaneh Assadian, post doc
Roberta Biasiotto, post doc
Anette Carlsson, technician
Wael Kamel, PhD student
Xin ”Susan” Lan, PhD student
Sara Östberg, PhD student

Project workers during 2014

Leo Nore (2 months)
Kwang-Chol Mun (6 months)
Elsa Willebrandt (3 months)

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CONTRIBUTION OF ADENOVIRUS INFECTIONS IN RECURRENT AND CHRONIC TONSILLITIS

Farzaneh Assadian

Surprisingly little is known about the pathogenesis of tonsillitis. To study the contribution of adenovirus infections in tonsillar diseases, I have established a patient-derived tonsil biobank (at present 100 patients), which allows us to classify particular clinical material according to the diagnosis, age and sex of the patients. This biobank is developed in collaboration with the clinicians at the Department of Surgical Sciences, unit of Otolaryngology, Uppsala University Hospital (Prof. Göran Laurell). During the last year I have set up efficient tonsil processing

methods, which allow me to prepare the high quality single-cell suspensions from tonsillar tissues. Thus, current experimental approach makes it possible to isolate pure fractions of B and T cells from tonsillar mononuclear cells (Assadian *et al*., manuscript submitted).
To assess the contribution of adenovirus infections in recurrent and chronic tonsillar diseases, I have planned the following experiments. First, purified B and T cell fractions will be characterized for the presence of adenovirus genomic DNA by qPCR. This approach will define the prevalence and serotype specificity of adenovirus infections in lymphocyte populations. In addition, I will take advantage of the Rolling-Circle Amplification (RCA)-based single molecule detection technique to visualize the presence of viral DNA at the single cell resolution. Second, I will analyze the presence and abundance of the viral derived miRNAs (the mivaRNAs) in the mentioned cell populations. For this experiments I have established a modified small RNA isolation protocol, which recovers high-quality RNA from the isolated cell fractions. To analyze the mivaRNAs, I will use a state-of-art RNA-sequencing method, which will reveal the origin and biochemical characteristics of the viral mivaRNAs in the T and B cell subpopulations. Further, the mivaRNA profiles will be correlated to the presence of viral DNA in the B and T cell populations. Finally, the quantitative data will be correlated to the patient's medical records.

REGULATION OF ADENOVIRUS EARLY PROTEIN E1A EXPRESSION BY THE VIRAL LATE PROTEIN L4-22K

Roberta Biasiotto

Human adenoviruses (Ad) have a double strand linear DNA genome, encoding for several early, intermediate and late proteins, subjected to a fine-tuned regulation at the transcriptional and post-transcriptional level, through differential polyadenylation and alternative RNA splicing.

The late protein L4-22K was shown to play a key role in the regulation of the viral gene expression: it stimulates the transcription from the major late promoter by a feed-forward mechanism and it is specifically involved in the splicing of the L4-33K mRNA. On the other hand, it affects the early genes expression and it is required for the packaging of Ad genome.

The aim of the present project is to characterize the potential role of the L4-22K protein as a regulator of the alternative splicing of the early E1A gene, a transcriptional activator responsible for the activation of the other viral early and several cellular genes. By alternative splicing, the primary E1A transcript produces five mRNAs (13S, 12S, 11S, 10S, and 9S) that are differentially expressed during the infection cycle.

By using a semi-quantitative PCR assay I demonstrated that L4-22K activated selectively 10S mRNA expression. A similar effect was also found for L4-33K, suggesting that the shared N-terminal region of these two proteins might be essential for this effect on E1A alternative mRNA accumulation. Further, I have shown that the packaging domain (containing binding sites for L4-22K, called A-repeats) in the E1A promoter region is not involved in the differential accumulation of the E1A isoforms induced by the L4-22K protein. Instead, the analysis of E1A mutants suggests that the minor intron of E1A might be the target region for the L4-22K-dependent regulation of E1A alternatively spliced mRNA accumulation. Further experiments aim at elucidating the mechanism through which L4-22K regulate E1A mRNA expression.

FUNCTIONAL CHARACTERIZATION OF VIRAL NON-CODING SMALL RNA FROM DIFFERENT ADENOVIRUS SEROTYPES.

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 the VA RNAs also suppresses the RNAi machinery in adenovirus-infected cells at multiple levels. Most importantly, the VA RNAs are processed by the cellular Dicer enzyme into virus-specific miRNAs, the so-called mivaRNAs. The mivaRNAs are efficiently incorporated into the RNA-Inducing Silencing Complexes (RISCs) in late virus infected cells. I have created the first model system where the predicted miRNA-like function can be specifically investigated without the interference of the other VA RNA functions. This was accomplished by construction of recombinant adenoviruses, in which the seed sequence of the mivaRNAI was mutated. The results showed that late viral protein synthesis as well as new virus progeny formation were essentially unaffected by the mivaRNAI seed sequence mutations under lytic growth conditions in HeLa or HEK293 cells. Thus, the result suggested that either strand of the mivaRNAI duplex does not have target mRNA interactions that are critical for the establishment of virus growth under lytic conditions in cell culture.

In addition, we have also utilized a phylogenetic conservation strategy to study the function of the mivaRNAs in different human adenovirus subgroups. We have shown that the terminal stem of the VA RNAs originating from human serotypes Ad4, Ad5, Ad11 and Ad37 all undergo Dicer dependent processing into mivaRNAs. We further demonstrated that the mivaRNA duplexes are subjected to a highly asymmetric RISC loading with the 3´-strand from all VA RNAs being the favoured strand, except for the Ad37 VA RNAII, where the 5´-mivaRNAII strand was preferentially assembled into RISC. Although the mivaRNA seed sequences are not fully conserved between the Ads a bioinformatics prediction approach suggests that a large fraction of the VA RNAII-, but not the VA RNAI-derived mivaRNAs are still able to target the same cellular genes. Future work will focus on experimentally validating the conserved targets between different serotypes, and investigations on the significance of these for the adenovirus life cycle

EFFECT OF THE FIRST LEADER 5' SPLICE SITE ON L4-22K-MEDIATED ACTIVATION OF THE ADENOVIRUS MAJOR LATE PROMOTER

Susan Lan

The adenovirus major late promoter (MLP) is responsible for synthesis of all essential mRNAs encoding the structural proteins of the viral capsid. The L4-22K protein is a multifunctional protein participating in different aspects of viral infection. One role is to function as an activator protein of MLP transcription. In this function L4-22K binds to the downstream elements (DE elements). I have shown that the intronic R1 element, spanning the major late first leader 5' splice site, has an inhibitory effect on L4-22K activation of MLP transcription. L4-22K binds to the distal part of the R1 region, a binding that stimulates the recruitment of at least three cellular factors to a site overlapping the first leader 5' splice site. One of these factors was identified as the cellular transcription factor Sp1. Binding of Sp1 to

the 5' splice site region had an inhibitory effect on L4-22K activation of MLP transcription *in vitro*. In addition, U1 snRNA binding to the first leader 5' splice site also appears to suppress L4-22K-mediated activation of MLP transcription both *in vivo* and *in vitro*. This finding suggests a novel coupling between MLP transcription and U1 snRNP interaction with the nascent MLP transcript. Future experiments are aimed at elucidating the mechanism(s) of L4-22K regulation of MLP gene expression.

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. Current work has generated an Oncolytic Virus patent, which has resulted in an early start-up company. The lab is now verifying the oncoselectivity of several alternative viral vectors in different models and also working on the initiation of human clinical trials.

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 want to study whether the function of the two proteins also are conserved. For this experiment we are testing 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.

VIRAL ZOONOSES

Åke Lundkvist, Erik Salaneck, Karin Sundström, Tanja Strand, Jenny Verner-Carlsson

Viruses have been with us since ancient times. They will also be our "companions" in the future, for as we have been able to defeat some diseases, new ones emerge or old ones re-emerge. Most human infections are zoonotic, meaning that they occur mainly in animals but also have the capacity to cross species-boundaries and attack humans.

Our research is based on an interdisciplinary approach between molecular virology, ecology, immunology, genetics, molecular epidemiology and diagnostic aspects of zoonoses, especially emerging zoonotic viruses. We are at present focusing on the following agents: hantaviruses, flaviviruses (TBE, Dengue and West Nile viruses), Sindbis virus and avian influenza virus.

Our hantavirus program has generated important results concerning novel animal models (monkey and rodents), vaccine candidates, virus-host interactions, pathogenesis, and innate immunity. We have also found valuable results on how, and under which circumstances, various Bunyaviruses are transmitted and survive outside their vectors and hosts. The recent awareness of Seoul hantavirus present in Swedish pet rats made us initiate a broad investigation of rats as carrier of various microorganisms pathogenic to man. We have developed a number of new methods for identification and characterization of genetic markers responsible for infectivity/pathogenicity and new techniques for studies on how hantaviruses infect their rodent reservoirs. During 2014 we discovered Seoul hantavirus in the Dutch wild rat population and a significant underdiagnosis of Puumala hantavirus infections in the Netherlands. Most recently, we found Puumala hantavirus in rodents trapped in Uppsala and outside Stockholm, i.e. far outside the previously known endemic areas. Our rat-project has further revealed the presence of the most severe variant of *Leptospira (interrogans* serovar Icterohaemorrhagiae) in Swedish city rats.

Our research on TBE virus has focused on molecular epidemiology of the virus in the Nordic countries and in the Baltic states. The recent increase of clinical cases in Sweden encouraged us to investigate the mechanisms behind and to create hypotheses explaining such emergence. The different virulence and pathogenesis of the three distinct substrains of TBEV (Western, Siberian and Far Eastern) has recently been investigated and confirmed in a novel bank vole model.

Dengue virus constitutes of four quite distinct virus types, dengue 1-4. Unique clinical virus strains from Cambodia, isolated from patients suffering of classical dengue, dengue hemorrhagic fever and dengue shock syndrome, respectively, have been characterized for phenotypic and genotypic differences *in vitro* and *in vivo*.

The awareness of highly pathogenic avian influenza virus repeatedly infecting man prompted us to establish efficient surveillance systems based on wild birds, and to initiate basic research aiming for a better understanding of the transmission and dramatic changes in virulence. A similar projects on West Nile virus have now been finalized.

Members of the group during 2014

Åke Lundkvist, professor, group-leader
Erik Salaneck, senior researcher
Karin Sundström, post-doc
Tanja Strand, post-doc
Irina Golovljova, post-doc (mainly in Estonia)
Olga Katargina, post-doc
Jenny Verner-Carlsson, microbiologist
Jenny Hesson, PhD student
Elina Rintala, PhD student
Frida Wennerholm, (master-student January-May)
Tove Hoflund, (master-student January-May, researcher)
Olivia Borg, project-student (fall 2014)

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EPIGENETIC CONTROL DURING ADENOVIRUS INFECTION

Tanel Punga

The role of infectious agents in the pathogenesis of human disease has received an increased awareness over the past decades. In particular, virus infections appear to be associated with a number of malignant and metabolic disorders.

Epigenetics is a rapidly growing research field that investigates alterations in gene expression caused by mechanisms other than changes in DNA sequence. Virus infections usually induce various epigenetic modifications to ensure optimal viral replication in the recipient cells. Therefore, our group is interested in understanding how a virus infection alters cellular gene expression patterns by introducing epigenetic changes. We use human adenovirus as a model system for our studies. Using this model system we aim to understand what kind of epigenetic changes associate with lytic and persistent virus infections both in the virus genome as well as in the host cell genome. Our special interest is there concentrated on adenovirus "histone-like" protein named as pVII. At present we are characterizing the biochemical properties and functions of the pVII protein as well as its interplay with various chromatin modifications. In addition we study the epigenetic mechanisms involved in the onset of a devastating neurological disease Friedreich ataxia.

Characterization of the causative disruption or dysregulation of normal epigenetic signaling pathways involved in disease development will broaden the general understanding disease pathogenesis and may also lead to the innovation of novel therapeutic applications.

Members of the group during 2014
Tanel Punga, PI
Sibel Ciftci, PhD student (until December)
Raviteja Inturi, PhD student
Helen Bergquist, PostDoc

Project workers during 2014
Phuoc My Nguyen (2 months)

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DISECTING EPIGENETIC CHANGES IN NEURODEGENERATIVE DISEASE FRIEDREICH ATAXIA (FRDA)

Helen Bergquist, 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.
Our ongoing studies are focused on the interplay between different chromatin modifications and expanded GAA repeats on FXN locus. Our ultimate aim of the project will be to specifically modify epigenetic pathways by novel chemical compounds and thereby enhance expression of the FXN protein in FRDA cells.

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 Cullin-3 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.

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.

ADENOVIRUS TYPE 12 INDUCED INTERFERON RESPONSE

Catharina Svensson

Human adenovirus type 12 (HAdV-12), in contrast to HAdV-2, displays a relatively low virulence and slow replication in cultured human cells, which is manifested by premature death of HAdV-12-infected cells. Whereas HAdV-2 induction of IFN-β expression is transient, HAdV-12-infected cells maintain high levels of IFN-ß expression, protein kinase R (PKR) activation and eIF-2α phosphorylation throughout the infectious cycle. The failure of the HAdV-12 virus-associated RNA (VA RNA) to prevent PKR activation appears to be a major underlying cause of the poor growth of HAdV-12. The importance of the IFN-inducible PKR kinase in restriction of HAdV-12 is supported by the enhanced growth in HeLa cells where PKR is knocked down. Finally, ectopic expression of HAdV-2 VA RNAI increases HAdV-12 hexon protein expression, further suggesting that the restricted growth of HAdV-12 is due in part to the inability of the virus to evade the antiviral host response because of insufficient VA RNA expression.

The strong antiviral response against HAdV-12 might be important for the host to prevent the establishment of long-lasting/persistent infections in humans. In a separate project, our group is analyzing establishment of persistent HAdV-5 infections in cultured B-cells, as well as the presence of adenovirus in lymphoid cells isolated after tonsillectomies (see project description by Tanel Punga). So far, HAdV-12 has not been found in human tonsills. In our current work, preliminary results indicate that HAdV-12 is unable to establish a persistent infection in B-cells. Upcoming experiments aims to determine whether this is due to inability to evade the antiviral defense of the infected B-cells.

Members of the group during 2014
Catharina Svensson, professor
Wu Chenjun, researcher
Maria Soultsioti, Erasmus student

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ONCOLYSIS OF TUMORS CAUSED BY T-ANTIGEN TRANSFORMED NORMAL MOUSE EPITHELIAL CELLS BY HUMAN ADENOVIRUS

Catharina Svensson, Staffan Johansson

Human adenovirus (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 cannot be observed. Thus, the development of HAdV for oncotherapy is hampered by the lack of suitable immunocompetent mouse model systems where the oncolytic efficacies can be determined. We recently identified a non-transformed mouse cell line (NMuMG) where the infection by HAdV2 is rapid and results in efficient production of new virus. Our results also showed that the NMuMG cells support growth of HAdV of types

D and E, but not of types A, B or F. In the follow-up 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. Preliminary results demonstrate that HAdV efficiently enters into both the cytoplasm and the nucleus, but that the expression of the immediate early gene E1A is undetectable in cells infected with restricted HAdV types.

The NMuMG cell line is derived from normal mouse mammary epithelial cells and retains the growth characteristics of non-transformed primary cells. Since NMuMG cells cannot form tumors in mice, we have established a tumorigenic NMuMG cell line expressing the T antigen of SV40 virus (NMuMG-T). Infection of NMuMG-T-induced tumors in mice by HAdV-2 efficiently reduced tumor growth and correlated with the ability of the virus to infect and replicate. So far, the oncolytic properties have been investigated in SCID mice. The current aim is to identify the origin of NMuMG cells to be able to repeat the analysis in immunocompetent, syngenic mice.

Members of the group during 2014

Catharina Svensson, professor
Staffan Johansson, professor
Zixuan Liu, master student
Xiaofang Cao, post doc
Wu Chengjun, researcher

Publications 2012 to 2014

1. Chengjun Wu, Daniel Öberg, Asif Rashid, Rajesh Gupta, Marco Mignardi, Staffan Johansson, Göran Akusjärvi and Catharina Svensson (2013) *A mouse mammary epithelial cell line permissive for highly efficient human adenovirus growth.* Virology 435, 363-371.

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DISSERTATIONS 2014

Johan Heldin: Identification and Characterization of Proteins and MicroRNAs that Modulate Receptor Signaling,Vesicular Trafficking and Cell Migration in Vascular Cells, February 14, 2014

Vahid Reyhani: Extracellular Matrix and Actin Cytoskeleton - The Control Unit of Interstitial Fluid Volume, May 9, 2014

Marlen Adler: Mechanism and Dynamics of Carbapenem Resistance in Escherichia coli, June 5, 2014

Julia Femel: Therapeutic Cancer Vacccines Targeting Molecules Associated with Tumor Angiogenesis, September 26, 2014

Nizar Enweji: Dynamics of resistant plasmodium falciparum parasites, November 12, 2014

Jonas Berglund: Meotic Recombination in Human and Dog: Targets, Consequences and Implication for Genome Evolution, November 20, 2014

Jessica Bergman: Genetics and growth regulation in Salmonella enterica, December 16, 2014

Erik Gullberg: Selection of resistance at very low antibiotic concentration, December 17, 2014

LICENCIATE THESIS 2014

Zhang Lu: Complement receptors 1 and 2 and IgG3 in immune responses and autoimmunity, November 6, 2014

Cifti Sibel: Molecular characterization of long-term adenovirus infection in B-cells, November 28, 2014

Xu Hui: Role of CD23 and CR1/CR2 in antigen transport, December 4, 2014

PRIZES AND AWARDS 2014

1) The Wolf Prize in Agriculture

The Wolf Prize is awarded annually since 1978 in the fields of Agriculture, Arts, Chemistry, Mathematics, Medicine and Physics by the Wolf Foundation in Israel. The Foundation began its activities in 1976 with a donation of $10 million by the Wolf family. The Foundation has a status of a private not-for-profit organization.

In 2014 Leif Andersson was awarded the prize in Agriculture "for providing groundbreaking contributions to plant and animal sciences, respectively, by using modern technologies of genomic research."

2) The Older Linné Medal in Gold (äldre Linnémedaljen i guld) by the Royal Academy of Sciences

In 2014 Ulf Lindahl, together with Sven-Olof Holmgren and Uno Lindberg, were awarded this medal in gold for their extraordinary efforts to strengthen school education in the area of natural sciences (för deras utomordentliga insatser för att stärka skolundervisningen inom naturvetenskaperna).

3) Uppsala University Pedagogic Prize in Medicine and Pharmacy

This prestigious pedagogic prize was awarded to Birgitta Tomkinsson in 2014 for her long time efforts to develop the "biomedicinska analytikerutbildningen".

Prize motivation: "*Birgitta Tomkinson är uppskattad av studenter och kollegor. Hon är en tydlig pedagogisk ledare, och arbetar ständigt för att förbättra undervisningen. Som ordförande i program-kommittén för biomedicinska analytikerprogrammet bidrar hon till en konkurrenskraftig utbildning baserad på en vetenskaplig grund. Birgitta Tomkinsons lyhördhet för studenternas åsikter leder till en dialog som påverkar kursutformningen. Hon är en lysande föreläsare och hennes undervisning är omväxlande, integrerande och mycket uppskattad.*"

UNDERGRADUATE TEACHING AT IMBIM

IMBIM has 18 full professors and associate professors as well as around 20 assistant professors and research fellows who contribute to the Department's undergraduate teaching. Additionally, there are some 40 PhD students who act as teaching assistants in the practical course work.

IMBIM participates in four different undergraduate programmes - Medicine, Pharmacy, Dispensing pharmacy, Biomedicine and Biomedical laboratory science - as well as two master programmes, one in Infection biology and the other in Medical Research. In all of these programmes, laboratory work is an important part and IMBIM has about 400 m^2 lab space dedicated for this purpose; some 20 different practicals are given by IMBIM each year, some of which are common to two or three of the programmes.

Medicine

In the Medical programme, which is 11 semesters long, each course focuses on a specific medical topic - rather than the specialisation of a department – and therefore teachers from different departments share the education duty in different courses. Thus, teachers from IMBIM take part in courses covering topics like ”Energy and nutrition balance”, ”Homeostasis and endocrine regulation” and ”Attack and defence”. 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. Around 110 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 the 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 teaching of biochemistry, cell biology, immunolo gy and microbiology.

Biomedical Laboratory Sciences

This 3-year programme leads to a Bachelor of Medical Science (Major in Biomedical Laboratory Science) that 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 specialize 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

MEDICAL RESEARCH

This two-year master programme aims to provide research-oriented students a sound preparation for PhD studies. During the first year students follow master courses offered by the medical faculty. The second year is dedicated to individual research practice carried out by rotation through different lab projects in parallel to following theoretical research-oriented topics. IMBIM manages the students during their rotation between different lab projects and the theoretical programme part.

INFECTION BIOLOGY

IMBIM is in charge of a two-year master programme in infection biology. During this period the student will meet a thorough education on microbes that surround us all the time and may endanger but potentially also help to treat diseases. With start from a molecular perspective the student will learn about diagnosis and therapies, development of resistance and surveillance. IMBIM is in charge of the programme, while some of the course modules are given in collaboration with other departments and faculties.

THE PhD PROGRAM AT IMBIM

During 2014 the department had 50 students registered for postgraduate studies. Eight defended their PhD theses and three students 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 helps new students with practical matters like employment, lodging and financial issues and to clarify what to expect from the department contra the responsibilities of the students. IPhAB also organizes regular social events during the semesters to increase the interaction between students and employees at IMBIM. The monthly IMBIM seminar series, which has been running for many years, has been substituted with two "IMBIM days", starting 2014. To broaden the knowledge of research conducted at IMBIM, researchers at the department will present their work for the whole department. Attendance at the IMBIM days is highly recommended for PhD students since they will generate the credit points needed to fulfil the requirements of a PhD. Further, the different research constellations at IMBIM have arranged seminar series specific for their respective scientific interests. During 2014, seminars in Bacteriology, Genomics, Immunology, Virology and Tumor Biology have been 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.

ECONOMY
(kSEK)

	2013	**2014**
Undergraduate Education Grant	22.852	21.507
Faculty Grant	69.561	42.105
External Grants	80.723	99.786
Others	1.042	1.167
Total	**174.178**	**164.565**

RESOURCE CENTER 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.

LIST OF AUTHORS