Institute of Biotechnology
Vilnius University

MoBiLi

SEVENTH FRAMEWORK PROGRAMME

Vilnius University Institute of Biotechnology

Biennial Report 2011–2012

Design by Vilnius University Press
Photographs by Giedrius Kuzmickas and the Institute of Biotechnology
Printed by Standartų spaustuvė

Institute of Biotechnology
Vilnius University

MoBiLi

Vilnius University
Institute of Biotechnology

Report
2011–2012

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Director's note

The Biennial report for 2011-2012 marks the first two years of the integration of Institute to Vilnius University. This period was complicated for several reasons. It was necessary to adjust to the new administrative rules and to overcome new bureaucratic challenges. In addition, this period coincided with the global economic crisis, which resulted in up to 40% reduction in budget of the Institute and reduced the competitive grant funding opportunities in Lithuania.

Auspiciously, in this difficult period we implemented the EC FP7 program project MOBILI directed to integration of the Lithuanian Modern Biotechnology into the European Research Area. Due to this project the scientists of the Institute had a lot of opportunities to actively participate in scientific conferences, invite famous lecturers to the Institute and let the internships visiting the Western European academic institutions. Owing to this project, seven experienced scientists, who had been previously working abroad, joined the Institute. They have successfully integrated into ongoing research topics of the Institute and in parallel started there new projects in most promising research fields. Thanks to this project the mobility of the research staff of the Institute has increased tremendously and contacts with foreign scientists have expanded.

The last two-year period was successful for participation in a wide range of applications for tenders. Our scientists carried out seven EC FP7 Programme projects, two NIH (USA) projects, and a HHMI (USA) project. In particular, scientists have been very active in the Research Council of Lithuania (LMT) and Agency for Science, Innovation and Technology (MITA) programmes. During this period of time our scientists started implementing 6 Global Grants financed by the European Structural Funds, 2 Swiss-LT projects funded by the Swiss Funds, 18 research team projects funded by LMT, 6 projects of National Programmes, one High-Technology Programme project and two grants of the Programme of Industrial Biotechnology. Both the National Integrated Programme of Biotechnology & Biopharmacy and the Programme of Industrial Biotechnology were initiated by the scientists of the Institute. These programmes have provided opportunities to purchase new equipment and materials, and help to integrate research and industry.

Scientific activity in competitions helped to overcome a crisis in 2009 and significantly improved the financial situation of the Institute. Scientists' activity, swiftness, high level internationally recognized results and their implementation in practice have influenced the next period funding, and our budget for 2013-2015 years has increased by about 24%.

The good news is that this period was an active patenting of research results and IP licensing. During the last two years the Institute published 5 patents, 3 of which were licensed to our neighbour Thermo Fisher Scientific.

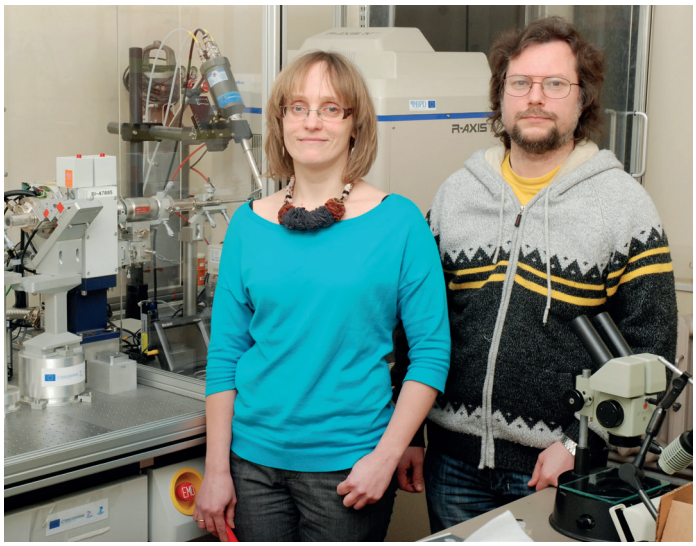
In my opinion, the most important landmark in the history of the Institute of Biotechnology is the creation of Lithuanian modern biotechnology industry, which is competitive on the world market. We take exceptional pride of our internationally recognized companies UAB Fermentas (currently Thermo Fisher Scientific), UAB Sicor Biotech (currently Teva), UAB Biocentras, UAB Biok, and the new UAB Profarma (2007), UAB Nomads (2010), UAB Baltymas (2011), UAB IMD Technologies (2012).

I hope you enjoy reading this report on significant developments and activities of the Vilnius University Institute of Biotechnology in 2011-2012.

Prof. Kęstutis Sasnauskas

Institute of Biotechnology: Just Facts and Numbers

- The Institute of Biotechnology was established in 1990 after restructuring of the All Union Research Institute of Applied Enzimology. Since October 1, 2010 it has become an internal unit of Vilnius University.
- Located at V.A. Graičiūno 8, Vilnius, Lithuania.
- Total staff number is 128; research staff number is 82, it includes 56 researchers (PhD).
- The youngest Lithuanian research institute - average age – 38.
- Allocation of state budget (2012) comprises 26% of income; other 74% comes from outside sources (grants, programmes, contracts).
- High level science along with applied research. Nine scientists of the Institute were awarded the Lithuanian Science Prize:
  prof. V.Butkus and prof. A. Janulaitis (1994),
  prof. S.Klimašauskas and prof. V. Šikšnys (2001),
  dr. A.Ražanskienė, dr. A. Gedvilaitė and
  prof. K. Sasnauskas (2003),
  dr. Č.Venclovas (2009),
  dr. D.Matulis (2012).
- Top level 25-30 scientific papers in peer reviewed high impact journals each year; coming patent applications; 5 patents published in 2011-2012, 3 of them were licensed to Thermo Fisher Scientific.
- Successful participation in EC (FW5, FW6, FW7) and other competitive international programmes (HHMI, NIH, EEA).
- A winner of the EC FW7 – Regional Research Potential: Coordination and Support action (FW7-REGPOT-2009—1) directed to the integration of European research entities into the European research area - MoBiLi project – 1.600.000 Euros.
- Selected as a Centre of Excellence in 2003 – EC FW5 programme - 600 000 Euros.
- Successful participation in projects of the *European Social Fund under the Global Grant Measure* – six projects – 2 501 200 Euros for 2011-2015.
- Since 2000 thirty researchers from abroad have joined the Institute.
- Involved in education of students at Vilnius University, Gediminas Technical University, Kaunas University of Technology. Part of the Institute lecturers are members of Committees on preparing Study Programmes.
- 25—30 students accomplish Bachelor or Master theses at the Institute each year.
- Thirty students are currently enrolled in Biochemistry or Chemical Engineering PhD studies at the Institute; all in all, nine PhD theses were defended in 2011-2012.
- Famous Lithuanian Biotech companies emerged from the Institute (UAB Fermentas (presently Thermo Fisher Scientific) - 1995, UAB Sicor - Biotech (presently TEVA) - 1995, UAB Biocentras - 1991, UAB Biok – 1991, UAB Profarma – 2007, UAB Nomads – 2010, UAB Baltymas – 2011, UAB IMD technologies – 2012.
- Skilful personnel for the Lithuanian Biotech are trained at the Institute; close connections with the Lithuanian Biotech industry are supported.
- National Industrial Biotechnology Programme was initiated by the Institute.
- National Integrated Programme of Biotechnology&Biopharmacy was initiated by the Institute.



Crystallographers Dr. Giedrė Tamulaitienė and Dr. Saulius Gražulis at an in house RIGAKU X-ray diffractometer

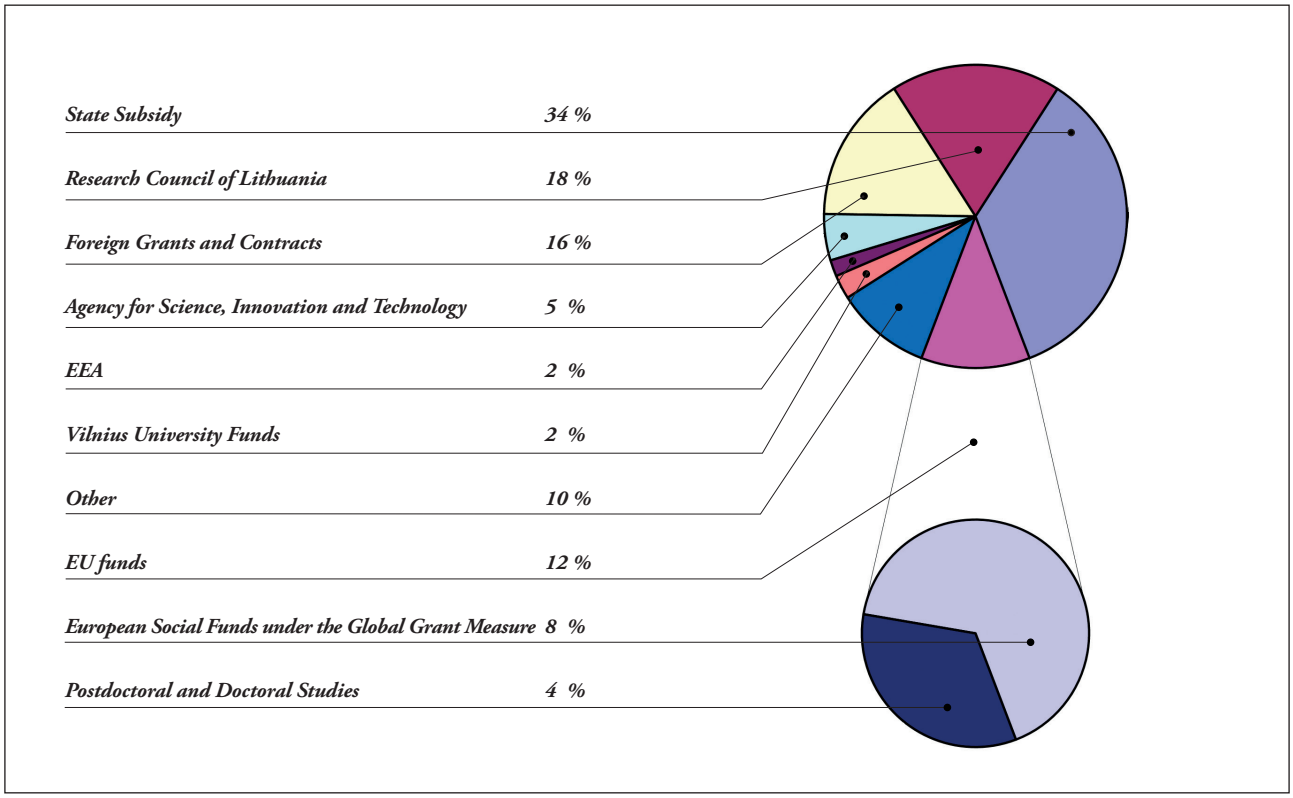
Undergraduate student Joana Gylytė performing isothermal titration calorimetry experiment

Dr. Arūnas Šilanskas and master student Skaistė Valinskytė purify proteins on ACTA avant system

Scientist Asta Zubrienė preparing samples for a biophysical protein-ligand interaction assay

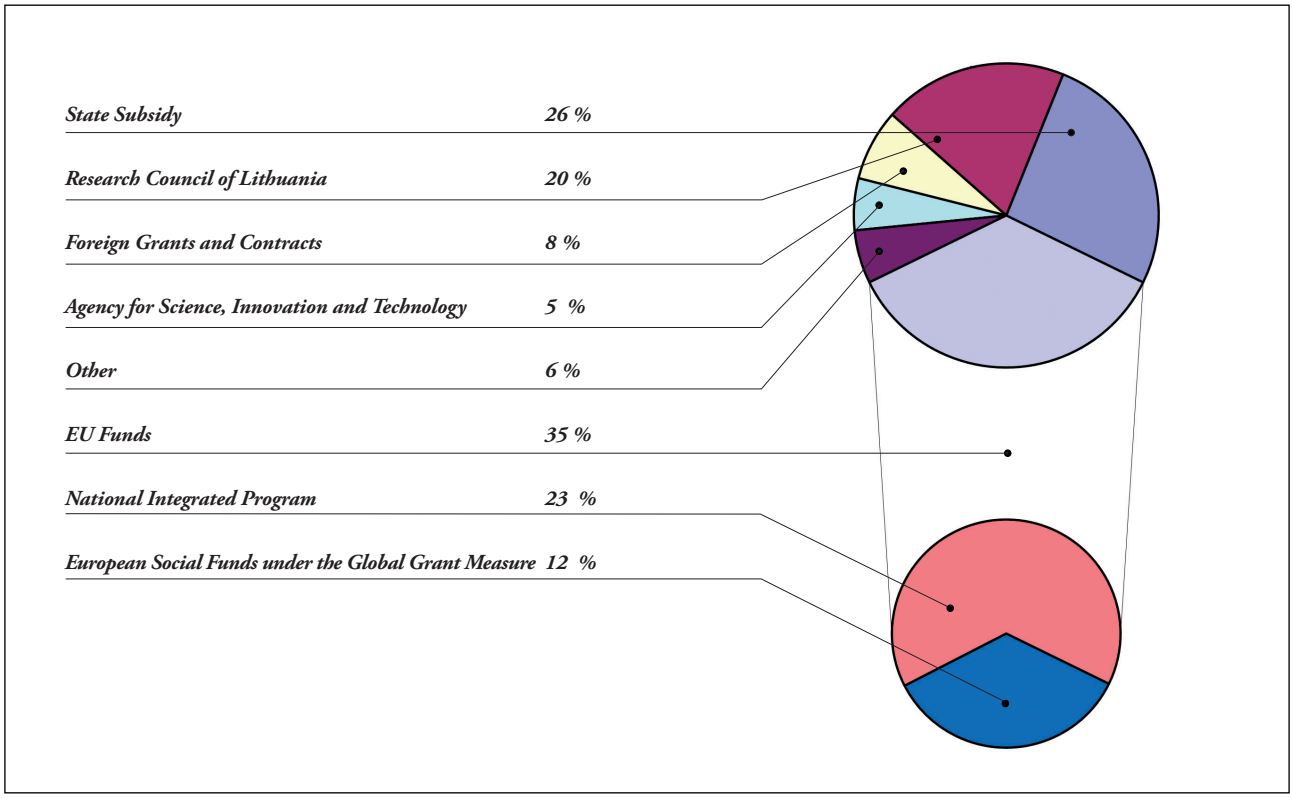
Financing Sources 2011
2.48 MEuros

	LTL thousands	EUR thousands
State Subsidy	3000.1	867.6
Research Council of Lithuania	1549.9	448.2
Foreign Grants and Contracts	1342.1	388.1
Agency for Science, Innovation and Technology	422.1	122.1
EEA	149.8	43.3
Vilnius University Funds	219.4	63.4
Other	871.1	251.9
European Social Funds under the Global Grant Measure	659.2	190.6
Postdoctoral and Doctoral Studies	332	96
	8545.7	**2471.2**



Financing Sources 2012
3.7 MEuros

	LTL thousands	EUR thousands
State Subsidy	3345.8	967.6
Research Council of Lithuania	2499.5	722.8
Foreign Grants and Contracts	969.7	280.4
Agency for Science, Innovation and Technology	705.4	203.9
Other	726.6	210.1
National Integrated Program	2940.9	850.5
European Social Funds under the Global Grant Measure	1596.2	461.6
	12784.1	**3696.9**



National and International Grants

EUROPEAN COMMUNITY GRANTS
Framework 7 programme

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Strengthening and sustaining the European perspectives of molecular biotechnology in Lithuania (MoBiLi)	L.Pašakarnis	5538.0	1603.9	2009-2013
Metastatic tumours facilitated by hypoxic tumour micro-environments (METOXIA)	dr. A.Kanopka	1289.6	373.5	2009-2013
Pan-European network for the study and clinical management of drug resistant tuberculosis (TB PAN-NET)	dr. P.Stakėnas	360.5	104.4	2008-2013
Development of novel antiviral drugs against Influenza (FLUCURE)	dr. G.Žvirblis	883.9	256.0	2010-2014
Integrated microfluidic system for long term cell cultivation, monitoring and analysis (BioCellChip)	dr. L.Mažutis	825.1	239.0	2012-2015
Towards construction of a comprehensive map of amyloid-ligand interactions: (-)-Epigallocatechin 3-Gallate and insulin amyloid (EGCG+INSULIN=)	dr. V.Smirnovas	345.3	100.0	2011-2015

OTHER INTERNATIONAL GRANTS
National Institutes of Health (USA)

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Approaches for genomic mapping of 5-hydroxymethylcytosine a novel epigenetic mark in mammalian DNA	prof. S.Klimašauskas	772.2	223.6	2010-2013

European Economic Area

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Anticancer drug design by structural biothermodynamics	dr. D.Matulis	1950.9	565.0	2008-2011

EU FUNDS National Integrated Programme

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Biotechnology and Biopharmacy: fundamental and applied research	prof. K.Sasnauskas	4926.4	1437.7	2012-2015

European Social Fund Under the Global Grant Measure

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Molecular tools for epigenomics and RNomics	prof. S.Klimašauskas	1576.0	456.4	2011-2015
Design of selective carbonic anhydrase, Hsp90, and Hsp70 inhibitors and investigation of their anticancer properties	dr. D.Matulis	1399.9	405.5	2012-2015
The use of genome-wide analysis for engineering of new yeast strains with improved heterologous expression	dr. R.Slibinskas	1309.3	379.2	2012-2015
Exploring flavones as universal inhibitors of amyloid-like fibril formation	dr. V.Smirnovas	1387.6	401.9	2012-2015
Structure and molecular mechanisms of bacterial antivirus defence systems	prof. dr. V.Šikšnys	1582.8	458.4	2011-2015
Novel chimeric proteins with antiviral activity	dr. A.Žvirblienė	1380.3	399.8	2012-2015

NATIONAL GRANTS
Research Council of Lithuania
National Research Programme "Chronic Non-infectious Diseases"

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Mutation, in Monoamine Oxidase B Gene, Which is Associated with Parkinson's Disease, Influence for Pre-Mrna Splicing	dr. A.Kanopka	147.2	42.6	2010-2011
Splicing factors and their regulated miRNA as cancer biomarkers for gastrointestinal system	dr. A.Kanopka	749.2	217.0	2012-2014
Carbonic Anhydrases as Cancer Cell Markers	dr. D.Matulis	294.8	85.4	2010-2011
Carbonic anhidrase hCA XII as a potential marker for cancer cells	dr. D.Matulis	590.5	171.0	2012-2014
Studies on genetic and molecular allergy mechanisms in the Lithuanian birth cohort	dr. A.Žvirblienė (partner of Vilnius University Faculty of Medicine)	474.2	137.3	2010-2011
Studies on genetic and environmental allergy risk factors in the Lithuanian birth cohort	dr. A.Žvirblienė (partner of Vilnius University Faculty of Medicine)	692.0	200.4	2012-2014
Molecular mechanisms in Alzheimer's disease	dr. A.Žvirblienė (partner of Lithuanian University of Health Sciences)	132.7	38.4	2012

National Research Programme "Healthy and Safe Food"

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Expression analysis of anthocyanin biosynthesis genes in horticultural plants	dr. V.Kazanavičiūtė (partner of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry)	680.4	197.1	2012-2015

Interspecific hybrids of orchard plant - a novel source of anthocyanins	dr. R.Ražanskas (partner of the Institute of Horticulture, Lithuanian Research centre for Agriculture and Forestry)	1299.3	376.3	2011-2014

Lithuanian-Swiss Cooperation Programme

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Directed evolution of computer engineered enzymes using droplet based microfluidics	prof. A.Janulaitis	2430.5	703.9	2012-2016
Signalling control of pathogen induced plant immunity	prof. I.Meškienė	2089.1	605.0	2013-2016

Research Team Projects

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Influence of ozone high concentration on viroid and plant host interaction	dr. A.Abraitienė	142.4	41.2	2011-2012
Synthesis of fluorine benzimidazole sulfonamides and analysis of their interaction with carboanhydrases	dr. V.Dudutienė	180.0	52.1	2011-2012
Crystallography Open Database - an open-access database of small molecule crystal structures	dr. S.Gražulis	179.4	52.0	2010-2011
The role of alternative pre-mRNA splicing for the lytic replication of Kaposi' Sarcoma Herpesvirus	dr. S.Laurinavičius	325.5	94.3	2012-2014
Structural studies of protein-nucleic acids complex in solution	dr. E.Manakova	168.7	48.9	2011-2012
CONDOR - an advanced protein homology detection method	dr. M.Margelevičius	126.8	36.7	2011-2012
High-throughput screening of antibody-secreting cells using droplet-based microfluidics	dr. L.Mažutis	339.9	98.4	2012-2014
Regulation of signal transduction in A.thialiana	dr. I.Meškienė	178.7	51.8	2011-2012
Analysis of the pathogenicity of Gardnerella vaginalis strains	dr. M.Plečkaitytė	130.0	37.7	2010-2011

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Studies on the mechanism of the cytolytic activity of the bacterial toxin vaginolysin	dr. M.Plečkaitytė	306.6	88.8	2012-2014
A Universal Method for Recombinant Synthesis of Selenoproteins	dr. R.Rakauskaitė	296.1	85.8	2012-2014
Analysis and diagnostics of new human Parvoviridea family viruses	prof. K.Sasnauskas	168.2	48.7	2011-2012
Structural and functional relationships in endonuclease evolution	dr. G.Sasnauskas	160.0	46.3	2010-2011
Structure and function of 5-methyl and 5-hydroxymethylcytosine-directed restriction endonucleases	dr. G.Sasnauskas	347.0	100.5	2012-2014
Stress response analysis on the proteome level in cells producing recombinant proteins	dr. R.Slibinskas	165.7	48.0	2011-2012
Looking for the origins of mammalian prion 'strains'	dr. V.Smirnovas	305.8	88.6	2012-2014
Bacteriopage T4 primosome	dr. G.Tamulaitienė	154.7	44.8	2011-2012
The role of nucleotide flipping in specific protein-DNA recognition	dr. G.Tamulaitis	160.0	46.3	2010-2011
Structure, classification and distribution of bacterial C family DNA polymerases	dr. Č.Venclovas	180.0	52.1	2011-2012
Application of interatomic contacts for the assessment of three-dimensional RNA structural models	dr. Č.Venclovas	43.4	12.6	2012-2013
Development and application of new methods for small RNAs analysis	dr. G.Vilkaitis	162.5	47.1	2010-2011
Studies on the biogenesis molecular mechanism of non coding RNAs in plants	dr. G.Vilkaitis	350.0	101.4	2012-2014
The link between structure and specificity within nucleases	dr. M.Zaremba	160.0	46.3	2010-2011
Function of a molecular motor in atypical restriction-modification system	dr. M.Zaremba	345.0	99.9	2012-2014
Development of recombinant antibodies against carbonic anhydrase	dr. A.Žvirblienė	348.7	101.1	2012-2014

AGENCY FOR SCIENCE, INNOVATION AND TECHNOLOGY
High-Technology Development programme 2011-2013

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
Development of microfluidics technology for monodisperse vesicles production and improved drug delivery	dr. L.Mažutis	300.0	89.9	2012

Industrial Biotechnology Programme 2011-2013

Title	*Head of the project*	*Financing LTL thousands*	*Financing EUR thousands*	*Duration*
The development of innovative biocatalytic stain remover	dr. I.Matijošytė	250.0	72.4	2012
Development of innovative biotechnology for oil base lubricant production	dr. I.Matijošytė	215.5	62.4	2012

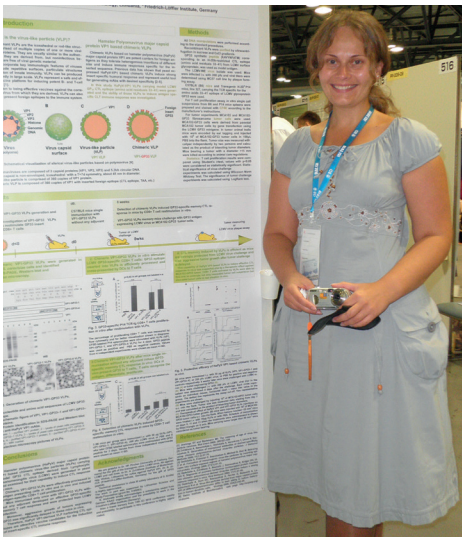
Doctoral theses

	Name	Title	Supervisor
2011	R. Gerasimaitė	A directed evolution design of target specificity and kinetic analysis of conformational transitions in the HhaI methyltransferas	prof. S.Klimašauskas
	I. Kučinskaitė-Kodzė	Production, characterization and application of new monoclonal antibodies against viral antigens	dr. A. Žvirblienė
	M. Juozapaitis	Synthesis of Paramyxoviridae nucleoproteins in yeast Saccharomyces Cerevisiae and their application in viral diagnostics.	prof. K. Sasnauskas
	E. Mažeikė	Generation of model anticancer vaccine based on virus-like particles	dr. A. Gedvilaitė
	E. Čiplys	Analysis of maturation of measles virus hemaglutinin in yeast S.cerevisiae and P.Pastoris secretory pathway and humanization of Yeast cells	dr. R. Slibinskas prof. K.Sasnauskas
2012	Z. Liutkevičiūtė	DNA cytosine methyltransferase-directed reactions involving non-cofactor-like compounds	prof. S.Klimašauskas
	A. Šilanskas	Restriction endonuclease-triplex forming oligonucleotide conjugates with controllable catalytic activity	prof. dr. V. Šikšnys
	D. Golovenko	Structural and functional studies of restriction endonucleases EcoRII, BfiI and Bse634I	dr. S. Gražulis prof. dr. V. Šikšnys
	G. Gasiūnas	Mechanism of DNA interference by Type II CRISPR/Cas systems	prof. dr. V. Šikšnys

Rūta Gerasimaitė enjoys a traditional gift from her colleagues

From left to right altogether Arūnas Šilanskas, Giedrius Gasiūnas, Zita Liutkevičiūtė and Dmitrij Golovenko who defended PhD theses at the Institute of Biotechnology in 2012

Just „released" doctor Dmitrij Golovenko with scientific consultants Dr. S.Gražulis (on left) and Prof. Dr. V. Šikšnys



Eglė Mažeikė at the 14th International Congress of Immunology in Kobe, August 2010

Indrė Kučinskaitė-Kodzė with the award of the Lithuanian Academy of Sciences for PhD theses in the field of biotechnology, 2012 March

Evaldas Čiplys enjoys the moment after defending his PhD theses

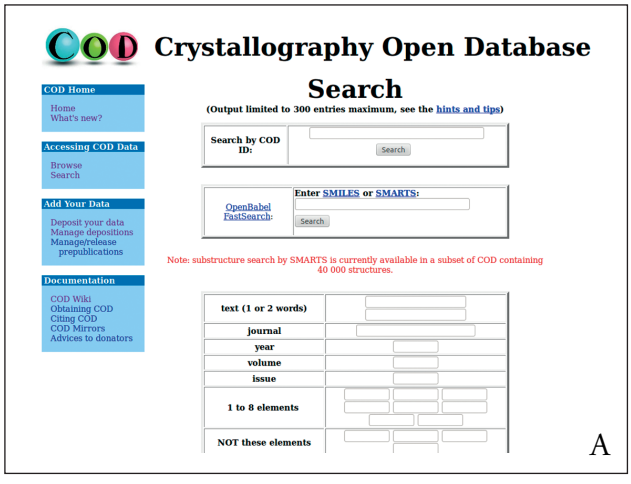
Crystallography Open Database (COD)

The COD project (abbreviated from the "Crystallography Open Database", http://www.crystallography.net/) aims at collecting in a single open access database all organic, inorganic and metal organic structures [1] (except for the structures of biological macromolecules that are available at the PDB [2]). The database was founded by Armel Le Bail, Lachlan Cranswick, Michael Berndt, Luca Lutterotti and Robert T. Downs in February 2003 as a response to Michael Berndt's letter published in the Structure Determination by Powder Diffractometry (SDPD) mailing list [3]. Since December 2007 the main database server is maintained and new software is developed in the Vilnius University Institute of Biotechnology by Saulius Gražulis and Andrius Merkys, and has now over 200 thousand records describing structures published in major crystallographic and chemical peer-reviewed journals [4]. Most of the mineral data is obtained from the AMCSD [5], donated by its maintainer and COD co-founder Robert T. Downs.

The database presents itself on the Internet as a Web site (Fig. 1A) with the basic data search and download capabilities, designed by Armel Le Bail and Michael Berndt. In addition, registered users may deposit new data into the database, either form the previous publications or as personal communications, using the deposition web site designed in the VU Institute of Biotechnology by Saulius Gražulis, Justas Butkus and Andrius Merkys. The deposition software performs rigorous checks of syntax and semantics, thus ensuring high quality of records deposited in the COD.

The retrieved COD records can be viewed on-line (Fig. 1B) or downloaded for further processing. For massive data mining, COD permits downloads and updates of the whole database using Subversion, Rsync or HTTP protocols. The ease of access to COD data has spurred the use of this resource for software testing [6], teaching [7], and research [8].

The open nature of the COD permitted numerous mirrors around the globe [9-12] and specifically tailored COD database variants [7]. At present, this is the most comprehensive open resource for small molecule structures, freely available to all scientists in Lithuania and worldwide.



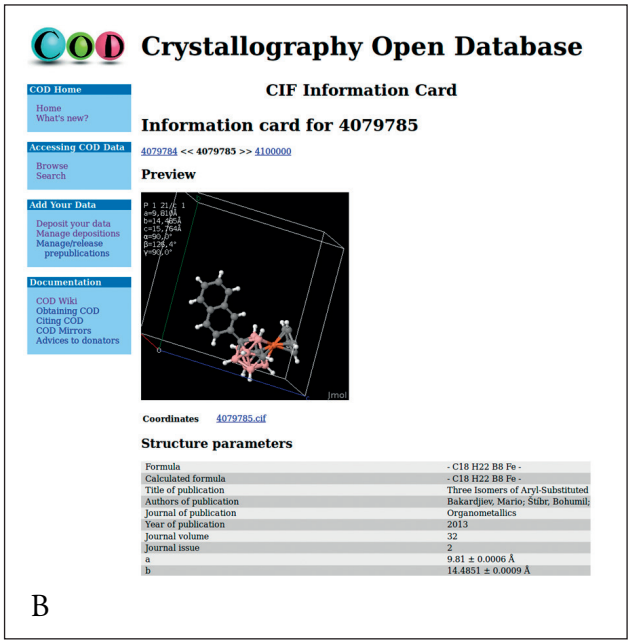


Fig. 1. A) Web site and search interface of the Crystallography Open Database (COD) permits searches of crystallographic data by a range of parameters and unrestricted retrieval of the found data. B) data can be viewed on-line in the JMol applet or downloaded for further processing, either record-wise or in bulk.

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Strengthening and Sustaining the European Perspectives of Molecular Biotechnology in Lithuania (MoBiLi)

MoBiLi is funded by the European Union, Research Potential Call FP7-REGPOT-2009-1

Mission of the MoBiLi:

MoBiLi is a support action to strengthen the research capacities and to mobilize human resources in molecular biotechnology at the Institute of Biotechnology (IBT) Vilnius, Lithuania. The MoBiLi, dedicated to the strengthening and sustaining the European perspectives of Molecular Biotechnology in Lithuania, has been selected for funding by the EU FP7 Capacities programme. The latter coordination and support action (call FP7-REGPOT-2009-1) was very competitive: 312 projects were received by the Commission and only 16 were selected for funding (MoBiLi ranked 7-th).

Purpose of the project

is to build up scientific excellence and human potential of IBT thereby transforming it into an excellence centre in molecular biotechnology and a significant player in the European Research Area.

The major objectives:

- Human capital building for research and technological development (RTD) in the field of state-of-the-art molecular biotechnology;
- Networking of IBT with major centres of excellence in the EU via joint research and mobility of researchers;
- Upgrading and modernisation of research infrastructure in line with emerging thematic priorities in the field.

The objectives of the project will be fulfilled by 7 Work Packages via collaboration with the project core partners:

The European Molecular Biology Laboratory (EMBL)
Karolinska Institutet, Stockholm (KI)
Justus Liebig University Giessen (JLU)
University of Edinburgh (UE)
The Swiss Institute of Bioinformatics (SIB)

Scientific priority areas of collaboration with the core partners cover topics like protein structure, interactions and cellular networks (JLU, EMBL, SIB, UE) and cellular imaging and high-throughput approaches to study human diseases (EMBL, KI, SIB, UE).

Project progress (October 2010 - December 2012)

Exchange of Know-How And Experience

The purpose of exchange programme is to strengthen the expertise and know-how of IBT.

During the period of 27 months, 12 scientists came to the IBT to do collaborative research and researchers from the IBT had made 46 visits to foreign partners.

Recruitment of Incoming Experienced Researchers

This work package includes measures for attracting researchers and establishment of new research trends.

Two group leaders, Prof. Irutė Meškienė and Dr. Linas Mažutis hired by IBT have established new research groups in line with the priority areas of MoBiLi. Projects carried out by these researchers are presented below.

Prof. Irutė Meškienė, PhD, Dr. Habil.

Biological functions of cell signaling components MAPK-phosphatases

During plant responses to stress or developmental cues signaling via protein kinases mediates fast, precise and specific responses in cells. Mechanism of signaling by the mitogen activated protein kinases (MAPK) is relatively clear, whereas termination of this process by the MAPK phosphatases is less understood.

The aim of our research is to understand the biological roles of PP2C-type MAPK phosphatases. Plant Arabidopsis thaliana provides excellent model for this study since its genome is sequenced and a variety of techniques are available to manipulate protein levels in plants.

We found that during cell signaling PP2C-type phosphatases are important to regulate MAPKs. This activity can influence cell fate decisions in stomata developmental pathway as well as plant sensitivity during pathogen attack.

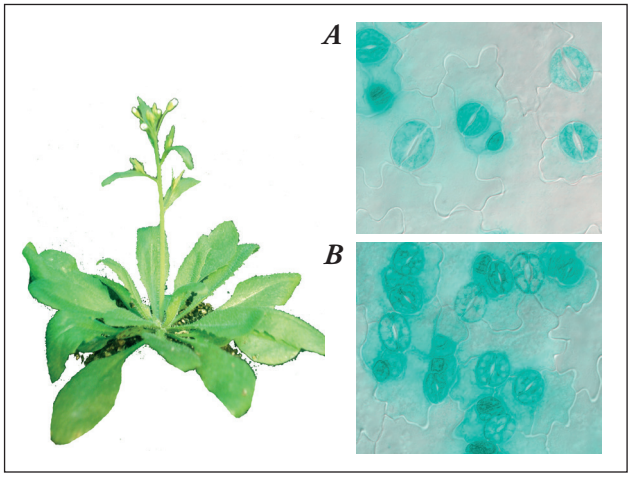

Figure 1. ***Study of PP2C-type MAPK phosphatase functions in Arabidopsis. Arabidopsis thaliana (right). Stomata development on leaf epidermal surface is affected by overexpression of the protein phosphatase AP2C3: A – wild type plant, B – AP2C3 overexpressing line (expression of the stomata cell-fate marker ERL::GUS, microscopy image 63x).***

Publications 2011-2013

1. Umbrasaite J., Schweighofer A., **Meskiene I.** Substrate analysis of Arabidopsis PP2C-type protein phosphatases. *Methods Mol. Biol.* 2011, 779:149-61.
2. Fuchs S., Grill E., **Meskiene I.**, Schweighofer A. Type 2C protein phosphatases in plants. *FEBS J.* 2013, 280(2):681-93.

Linas Mažutis, PhD

High-throughput screening using droplet-based microfluidics

Microfluidics technology has revolutionized many areas of science by providing unprecedented liquid-handling capabilities, reduced reaction volumes and improved analytical sensitivity. In droplet-based microfluidic systems, nanolitre to picolitre volume aqueous droplets in an inert carrier oil are used as microreactors with volumes one thousand to one million times smaller than microtitre plate wells, where the smallest reaction volume is a few microlitres. These droplets can be made, fused, split, incubated and manipulated in sophisticated ways at kHz frequencies. Compartmentalization of single molecules or cells into pico- or nano-liter volume droplets allows millions of individual reactions to be analyzed and sorted at hight-throughput rate. A particular advantage is that droplets provide a unique tool of linking genotype with phenotype through compartmentalization. For example, when using cells, secreted molecules remain entrapped inside the droplet together with the cell that produce them. When using genes, synthesized proteins remain entrapped together with the gene that encodes them. Microfluidic fluorescence activated droplets sorters can then be used to identify and sort entire reaction vessels containing in-

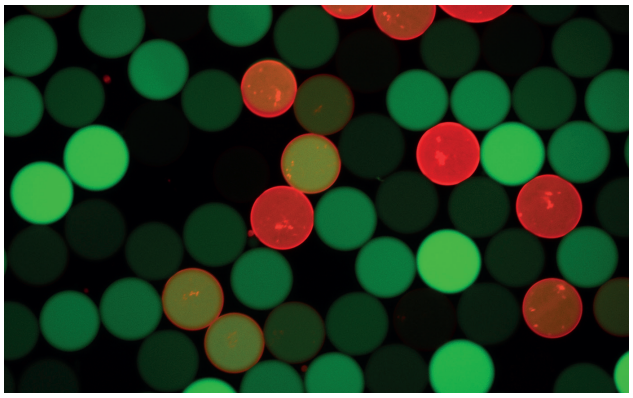

Figure 2. Microfluidic droplets having different enzyme variants produced and secreted by encapsulated bacterial cells.

dividual cells or molecules such as genes, proteins, RNA, etc. In our laboratory we are using droplet-based microfluidic systems for high-throughput analysis and screening in biology and chemistry. Current projects are focusing on directed evolution of computer-designed enzymes, screening of single-cells secreting specific antibodies and developing advanced drug delivery particles for biomedical applications.

Publications 2011-2012

1. Pekin D., Skhiri Y., Baret J.C., Le Corre D., **Mazutis L.**, Salem C.B., Millot F., El Harrak A., Hutchison J.B., Larson J.W., Link D.R., Laurent-Puig P., Griffiths A.D., Taly .V Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. *Lab Chip* 2011, 11(13):2156-66.
2. **Mazutis L.**, Griffiths A.D. Selective droplet coalescence using microfluidic systems. *Lab Chip* 2012, 12(10):1800-6.
3. Skhiri Y., Gruner P., Semin B., Brosseau Q., Pekin D., **Mazutis L.**, Goust V., Kleinschmidt F., Harrak A.E., Hutchison J. B., Mayot E., Bartolo J.-F, Griffiths A.D., Taly V. and Baret J.-C. Dynamics of molecular transport by surfactants in emulsions. *Soft Matter* 2012, 8(41):10618-10627.

5 experienced researchers

(postdoctoral associates)

Dr. Rasa Rakauskaitė, Dr. Vytautas Smirnovas, Dr. Simonas Laurinavičius, Dr. Visvaldas Kairys, Dr. Ieva Mitašiūnaitė-Besson had been hired to join the existing laboratories with the goal to strengthen their scientific potential. Projects carried out by these researchers are presented below.

Simonas Laurinavičius, PhD

Towards understanding the pathogenicity of Kaposi' Sarcoma herpesvirus

In my work I aim to combine my previous experience with the new ways to investigate the mechanisms of the diseases associated with Kaposi Sarcoma Herpesvirus (KSHV). The Department of Immunology and Cell Biology I am working in is experienced in studying regulation of gene expression at the level of pre-mRNA splicing, and is specifically interested in hypoxia-induced pre-mRNA splicing. In my project „The role of alternative pre-mRNA splicing for the lytic replication of Kaposi' Sarcoma Herpesvirus", funded by the Research Council of Lithuania, I want to investigate whether the pre-mRNA splicing status affects the induction of lytic replication of KSHV (lytic reactivation). The hypothesis behind this is that the cells that undergo spontaneous lytic reactivation in sub-population of cell lines or in Kaposi sarcoma tumors might have a different set of molecular splice-isoforms and that these differences influence the switch between the latent and lytic phases of replication cycles. As different environmental factors (such as hypoxia) or chemicals induce varying response of lytic reactivation of KSHV, it of interest to investigate, if these factors affect different populations of KSHV-infected cells. As it is know that certain proteins that are involved in pre-mRNA splicing are important in KSHV replication cycle, in my project I would also like to identify splicing-associated factors that might be responsible for the differences of the putative sub-populations of cells.

Rasa Rakauskaitė, PhD

Engineering of selenium-containing methyltransferases

DNA methylation is carried out by AdoMet-dependent methyltransferases and serves to expand the information content of the genome. DNA cytosine-5 methylation is a key epigenetic signal in higher eukaryotes including humans, and its misregulation is implicated in a number of syndromes and pathologies such as cancer. DNA cytosine-5 methytransferases (C5-MTases) use a conserved cysteine residue for covalent catalysis of the methyl transfer to their target cytosine residues in DNA. Apart from their important biological roles, C5-MTases are now increasingly exploited for biotechnological applications. Owing to the recently discovered atypical reactions involving synthetic cofactor analogs and small non-cofactor like compounds, C5-MTases can be used for targeted labeling of cytosine and 5-hydroxymethylcytosine in DNA and are potentially valuable tools for genome-wide analysis of the epigenetic cytosine modifications. To explore the catalytic power of these enzymes we set out to replace the catalytic cysteine residue with a selenocysteine (Sec) residue in a model C5-MTase, M.HhaI. Sec provides selenium atom with unique chemical characteristics (higher nucleophilicity, lower pKa, and lower redox potential) not attainable in common proteins. Incorporation of a Sec residue has been achieved through UAG codon suppression by the genetically encoded unnatural amino acid (Fig.3).

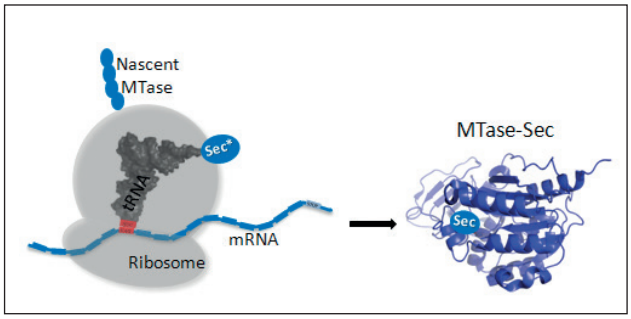

Figure 3. ***Incorporation of genetically encoded Sec residue into a MTase. The unnatural Sec residue is incorporated at a preselected position of a protein. During translation in yeast cells an engineered UAG codon is decoded by an artificially designed UAG suppressor tRNA charged with the unnatural amino acid.***

Visvaldas Kairys, PhD

Exploring interactions and dynamics of biologically important molecules using computational tools

One of the greatest challenges of computational chemistry is a correct evaluation of the non-bonded interaction strengths between small molecules and proteins. This requires employment of a wide range of theoretical methods, from quantum mechanics to docking and molecular dynamics (MD). The recent examples of computational chemistry research done at the Department of Bioinformatics are listed below.

Docking

In a recent study published in a collaboration with the University of Madeira (Portugal), a challenging docking of a highly charged oligomer onto a DNA fragment[1] was explored. The study showed that docking could lead to meaningful results even with a very large (>100) number of rotatable dihedrals in the ligand (Fig. 4).

Enzyme inhibitor discovery

In a close collaboration with the team at the Department of Biothermodynamics and Drug Design at IBT, the investigation is being carried out aimed at designing novel Carbonic Anhydrase (CA) drug-like inhibitors. Prediction of isoform selectivities towards clinically useful CA variants presents an additional challenge. A variety of computational methods are employed, including docking[2].

Molecular Dynamics

Presently, a significant portion of time is also devoted to the investigation of the protein dynamics (for example, human chorionic gonadotropin, DNA clamps) using MD simulations[3].

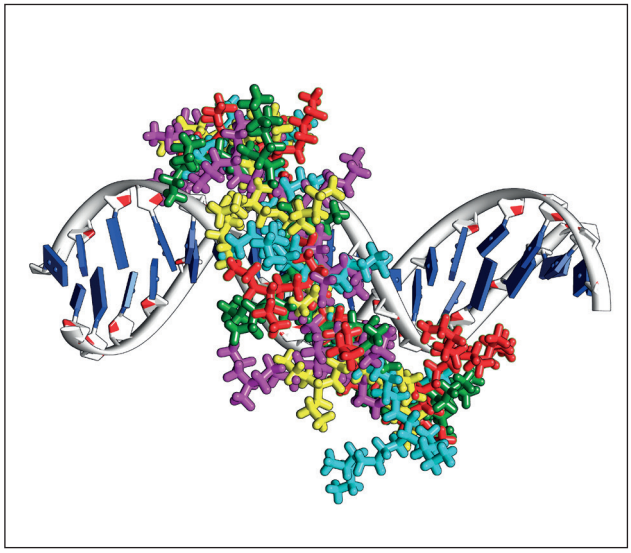

Figure 4. Five superposed best hits of poly(ethylenimine) trimer (PEI) docked to a DNA fragment. A a tendency to bind to the minor groove of DNA is observed.

Publications 2012

1. Nouri A., Castro R., **Kairys V.**, Santos J. L., Rodrigues J., Li Y., Tomás H. M. Insight into the role of N,N-Dimethylaminoethyl methacrylate (DMAEMA) conjugation onto poly(ethylenimine): cell viability and gene transfection studies. *J. Mat. Sci. Mater. Med.* 2012, 23:2967-2980.
2. Capkauskaitė E., Zubrienė A., Baranauskienė L., Tamulaitienė G., Manakova E., **Kairys V.**, Gražulis S., Tumkevičius S., Matulis D. Design of [(2-pyrimidinylthio) acetyl]benzenesulfonamides as inhibitors of human carbonic anhydrases. *Eur. J. Med. Chem.* 2012, 51:259-70.
3. Nagirnaja L., Venclovas C., Rull K., Jonas K.C., Peltoketo H., Christiansen O.B., **Kairys V.**, Kivi G., Steffensen R., Huhtaniemi I.T., Laan M. Structural and Functional Analysis of Rare Missense Mutations in Human Chorionic Gonadotropin β-subunit. *Mol. Hum. Reprod.* 2012, 18(8):379-390.

Vytautas Smirnovas, PhD

Studies of amyloid-like fibril formation

Protein aggregation and amyloidogenesis are involved in a number of diseases, including such neurodegenerative disorders as Alzheimer's and Parkinson's, many systemic amyloidoses and even some localized diseases such as type II diabetes or cataracts. There is an increasing evidence of amyloid nature of proteinaceous infectious particles – prions. Possible way of abnormal protein spreading is elongation of amyloid-like fibrils, thus there is a chance of all amyloid-associated diseases to be potentially infective.

We are interested in better understanding of fibril elongation. Using insulin as a model protein we employed Michaelis-Menten equation to describe fibril elongation kinetics (Fig.5).

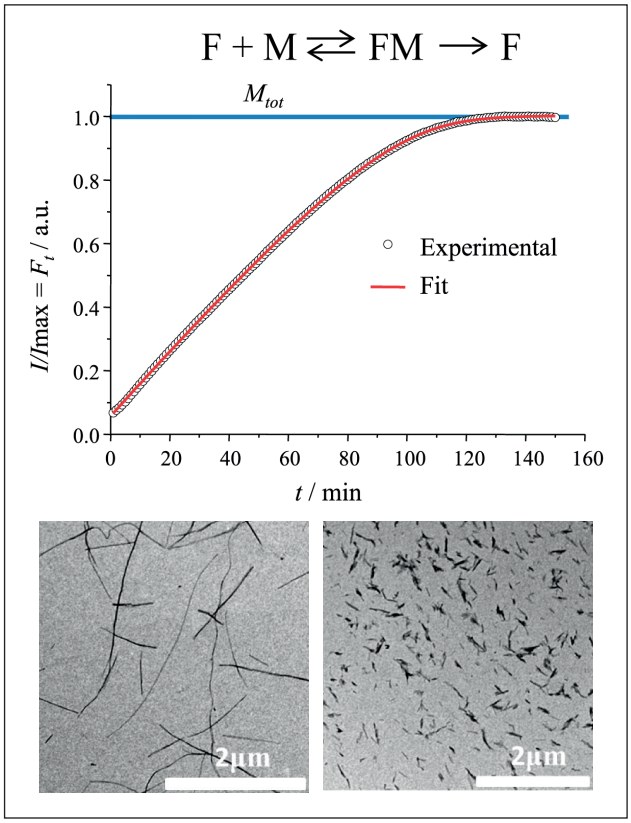

Figure 5. Left panel shows possible mechanism of fibril elongation (F stands for fibril, M – for monomer, and FM is a short-living complex, which exists from the moment when monomer attaches to the fibril until it's completely incorporated) and fit of the experimental elongation data using Michaelis-Menten equation. Right panel shows amyloid-like fibrils before (top) and after (bottom) ultrasonic treatment.

At this point we try to compare elongation thermodynamics for different amyloid fibrils at varying conditions, which may give a better understanding on protein-only infectivity.

Other side of our interest isamyloid-ligand interactions. We try to explore potential anti-amyloidogenic compounds and study their impact on fibril formation of different proteins.

Acquisition, Development, Maintenance or Upgrading of Research Equipment

The MoBiLi project is aimed to create a stimulating, multidisciplinary environment promoting research of excellence in biomedicine at the interface between structural biology, chemistry and biology. Therefore IBT has purchased the following equipment: Universal X-Ray Diffractometer, Gemini PX Ultra system, HPLC-MS System, Cell sorting system for high performance analytical and preparative flow cytometry and High performance computing (HPLC) Linux cluster. Total value is 600.000 Euro.

International Seminars & Workshops

The aim of this WP is to increase the international visibility of IBT, dissemination of scientific information obtained at IBT and exchange of know-how with potential collaboration partners. 18 experienced researchers including Nobel Prize winner Prof. Robert Huber, had visited IBT and gave their presentations. Furthermore, IBT researchers had attended 29 international conferences and workshops on structural and computational biology and biomedicine:

Dissemination and Promotional Activities

Dissemination activities will facilitate dissemination and transfer of knowledge at regional, national and international level involving both the own research/PR staff and invited specialists from other countries and will increase the international knowledge/experience exchange capacity and reputation of IBT. They will not only provide general information about MoBiLi and IBT as a whole, but will bring MoBiLi and IBT to an eyelevel position for future collaboration in

Prof. Ralf Seidel from the University of Technology in Dresden at his seminar* „Magnetic tweezers: from single enzyme dynamics to mechanics of DNA nanostructures“ *in VU Institute of Biotechnology on October 5, 2012

Prof. Pietro Speziale from the University of Pavia at his seminar on* „Biochemical and immunological properties of surface/secreted proteins of *Staphylococcus aureus* and their use as potential components for a multivalent vaccine” *in VU Institute of Biotechnology on September 19, 2012

research, e.g. EU FP7 as well as regional and national programs. MoBiLi had communicated its activities through a variety of communication channels, including:

- Publication of 10 open-access articles in scientific journals
- Instant highlighting of research achievements on the IBT web page mobili.ibt.lt and with the media
- Production of reports on IBT research achievements and services offered to the community in connection with the MoBiLi project
- Meeting with local biotech SME (UAB Sorpo, UAB Profarma, UAB Biocentras, UAB Fermentas) and Lithuanian Biotechnology Association (LBTA)
- Meeting in the Diagnostic Centre of Vilnius University Hospital

Announcements of the MoBiLi seminars are distributed to the target groups. A MoBiLi website (http://www.mobili.ibt.lt/) has been launched and is constantly updated. There is a link to the MoBiLi website at the IBT's site (http://www.ibt.lt/en/title.html) as well. Three articles with acknowledgements to the MoBiLi project had been published.

External Evaluation

To check and control the achieved research quality and scientific excellence at the project's end, an independent evaluation will be implemented. External evaluation facility is foreseen to take place after the end of the implementation in order to evaluate the applicant's overall research quality and capability (including management and infrastructure). Three experts had already been appointed by the Commission and the fourth had been contacted. The appointed experts will visit the applicant institution to discuss with the researchers and the research management in order to evaluate the capacity of the applicant to handle its objectives with the means available in situ and the perspectives to maintain or to increase the applicant's research capacity and the means necessary for this purpose.

From left to right the VU IBT International Advisory Board Members Prof. B. Samuelsson, Prof. L. Poellinger, Prof. A. Pingoud, Prof. S. Halford, Prof. H. Grosjean at the MoBiLi meeting in Vilnius University on May 6, 2011

Project Management

To ensure successful implementation and professional administration, vigorous and excellent project management is necessary. A second project's Advisory Board meeting took place on May 6-th, 2011. The Advisory Board members had met the recruited personnel and listened to their presentations. Their report had contributed largely to the success of changing the deadlines of implementation of workpackages 1-5 from November 2012 to May 2013. The Advisory Board members are Prof. A. Tramontano (University of Rome "La Sapienza", Italy), Prof. A. Pingoud, (Justus-Liebig-Universität, Germany), Prof. L. Poellinger, (Karolinska Institutet, Sweden), Prof. S. Halford, (University of Bristol, U.K.), Prof. B. Samuelsson, (University of Gothenburg, Sweden), Prof. H. Grosjean, (University of Paris-South, France), Prof. E. Butkus, (Research Council of Lithuania), Mr. A. Markauskas, (Fermentas, CEO, Lithuania), Dr. A. Žalys, (Ministry of Education and Science, Lithuania), Prof. G. Dienys, (Lithuanian Biotechnology Association). The Management Board members are Mr. Leonas Pašakarnis, (Deputy Director of Institute of Biotechnology Vilnius), Prof. Saulius Klimašauskas, (Head of Department of Biological DNA modification), Dr. Daumantas Matulis, (Head of Department of Biothermodynamics and Drug Design), Dr. Gintautas Žvirblis, (Head of Department of Eukaryote Genetic Engineering), Prof. Aurelija Žvirblienė, (Head of Department of Immunology), Dr. Česlovas Venclovas, (Head of Department of Bioinformatics), Prof. Virginijus Šikšnys, (Head of Department of Protein-Nucleic Acids Interactions).

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Rokas Grigaitis
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Irmantas Mogila

Research overview

Bacterial viruses (bacteriophages) provide an ubiquitous and deadly threat to bacterial populations. To survive in hostile environments, bacteria have developed a multitude of antiviral defense systems. The overall research theme in our department is the structural and functional characterization of enzymes and enzyme assemblies that contribute to the bacteria defense systems which target invading nucleic acids. In particularly, we are involved in the deciphering structural and molecular mechanisms of restriction enzymes, and the molecular machinery involved in the CRISPR function. We are using X-ray crystallography, mutagenesis, and functional biochemical and biophysical assays to gain information on these systems.

Structure and function of restriction endonucleases

Restriction-modification (RM) systems commonly act as sentries that guard bacterial cells against invasion by bacteriophage. RM systems typically consist of two complementary enzymatic activities, namely restriction endonuclease (REase) and methyltransferase (MTase). In typical RM systems REase cuts foreign DNA but does not act on the host genome because target sites for REase are methylated by accompanying MTase. REases from 4000 bacteria species with nearly 350 distinct specificities have been characterised. REases have now gained widespread application as indispensable tools for the *in vitro* manipulation and cloning of DNA. However, much less is known about how they achieve their function.

In the Department of Protein-Nucleic Acids Interactions we focus on the structural and molecular mechanisms of restriction enzymes. Among the questions being asked are: How do the restriction enzymes recognize the particular DNA sequence? What common structural principles exist among restriction enzymes that recognize related nucleotide sequences? How do the sequence recognition and catalysis are coupled in the function of restriction enzymes? Answers to these questions are being sought using X-ray crystal structure determination of restriction enzyme-DNA complexes, site-directed mutageneses and biochemical studies to relate structure to function (see below for the details).

Structure and molecular mechanisms of CRISPR-Cas systems

Recently, an adaptive microbial immune system CRISPR (clustered regularly interspaced short palindromic repeats) has been identified that provides acquired immunity against viruses and plasmids. CRISPR represents a family of DNA repeats present in most bacterial and archaeal genomes. CRISPR loci usually consist of short and highly conserved DNA repeats that are interspaced by variable sequences of constant and similar length, called spacers. CRISPR arrays are typically located in the direct vicinity of cas (CRISPR associated) genes. Cas genes constitute a large and heterogeneous gene family which encodes proteins that often carry functional nucleic-acid related domains such as nuclease, helicase, polymerase and nucleotide binding. The CRISPR-Cas system provides acquired resistance of the host cells against bacteriophages. In response to phage infection, some bacteria integrate new spacers that are derived from phage genomic sequences, which results in CRISPR-mediated phage resistance. Many mechanistic steps involved in invasive element recognition, novel repeat manufacturing, and spacer selection and integration into the CRISPR locus remain uncharacterized (see below for the details).

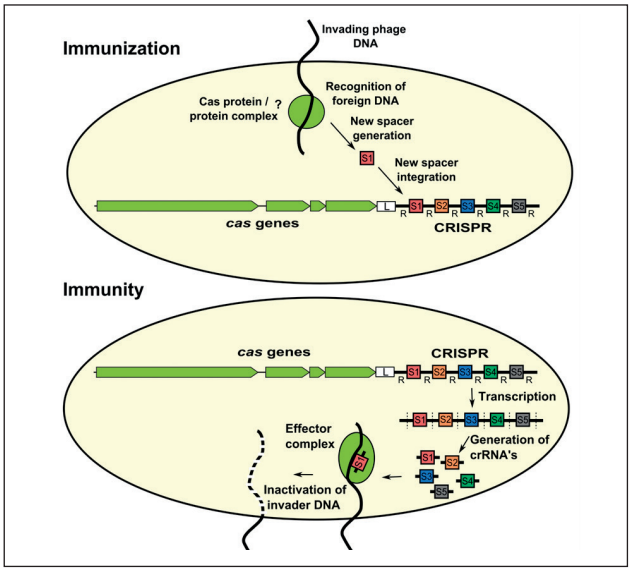

Figure 1. ***CRISPR-Cas system. CRISPR loci consist of short and highly conserved DNA repeats (R) interspaced by variable sequences of constant and similar length, called spacers (S). CRISPR repeat-spacer arrays are typically located in the direct vicinity of cas (CRISPR-associated) genes. In the immunisation steps, it is proposed that Cas proteins incorporate foreign DNA as new spacer sequences. This is a precise process that adds spacers of similar length to one end of the repeat. Thus, the repeat series acts as a historical record of viral infections. In the immunity step, it is proposed that RNA from the repeat region is produced and processed by Cas proteins to produce short signal RNAs. These crRNAs are then used to specifically target invading DNA for degradation.***

Structure and function of restriction endonucleases: projects overview

The recognition domain of the methyl-specific endonuclease McrBC flips out 5-methylcytosine

DNA cytosine methylation is a widespread epigenetic mark. Biological effects of DNA methylation are mediated by the proteins that preferentially bind to 5-methylcytosine (5mC) in different sequence contexts. Until now two different structural mechanisms have been established for 5mC recognition in eukaryotes; however, it is still unknown how discrimination of the 5mC modification is achieved in prokaryotes. To address this question we have solved the crystal structure of the N-terminal DNA-binding domain (McrB-N) of the methyl-specific endonuclease McrBC from *Escherichia coli*.

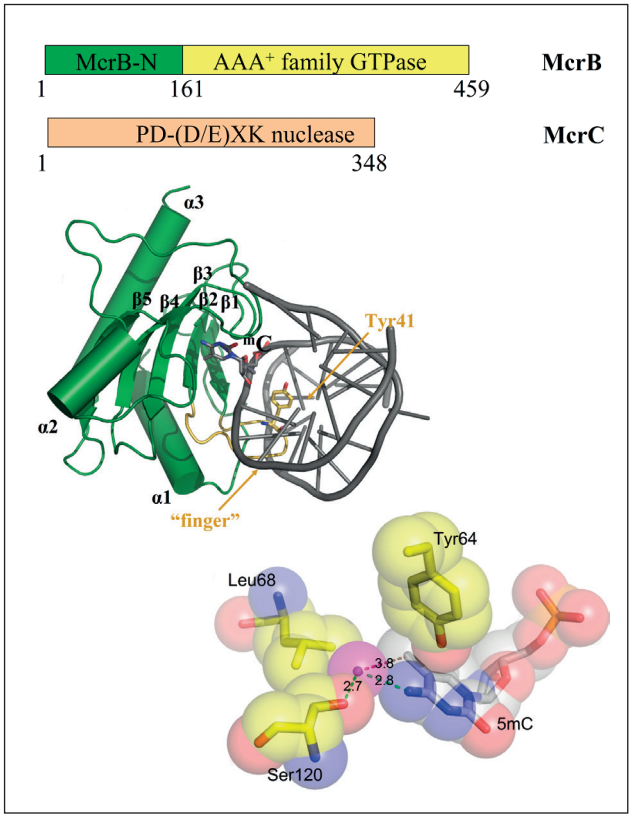

Figure 2. ***5-methylcytosine recognition by McrB-N. Top: schematic representation of the McrBC restriction system. McrBC is composed of two subunits: McrB harbours an N-terminal DNA-binding domain (McrB-N) and GTP-ase motifs, while McrC contains a nuclease active site. Bottom left: view of the McrB-N monomer bound to DNA. The 'finger' loop penetrating into the minor groove is highlighted in orange. Bottom right: Close-up view of the water molecule in the vicinity of the methyl group in the McrB-N complex with di-methylated DNA. The hydroxyl group of 5hmC or methyl group of the N4-methylcytosine may occupy the space filled by the water molecule (shown in magenta).***

The McrB-N protein shows a novel DNA-binding fold adapted for 5mC-recognition. In the McrB-N structure in complex with methylated DNA, the 5mC base is flipped out from the DNA duplex and positioned within a binding pocket. Base flipping elegantly explains why McrBC system restricts only T4-even phages impaired in glycosylation [Luria, S.E. and Human, M.L. (1952) A nonhereditary, host-induced variation of bacterial viruses. J. Bacteriol., 64, 557-569]: flipped out 5-hydroxymethylcytosine is accommodated in the binding pocket but there is no room for the glycosylated base. The mechanism for 5mC recognition employed by McrB-N is highly reminiscent of that for eukaryotic SRA domains, despite the differences in their protein folds.

Structural and functional studies of the Bse634I specificity

Restriction endonuclease Bse634I recognizes and cleaves the degenerate DNA sequence 5'-R/CCGGY-3' (R stands for A or G; Y for T or C, '/' indicates a cleavage position). We have solved the crystal structures of the Bse634I R226A mutant complexed with cognate oligoduplexes containing ACCGGT and GCCGGC sites, respectively. In the crystal, all potential H-bond donor and acceptor atoms on the base edges of the conserved CCGG core are engaged in the interactions with Bse634I amino acid residues located on the α6 helix. In contrast, direct contacts between the protein and outer base pairs are limited to van der Waals contact between the purine nucleobase and Pro203 residue in the major groove and a single H-bond between the O2 atom of the outer pyrimidine and the side chain of the Asn73 residue in the minor groove. Structural data coupled with biochemical experiments suggest that both van der Waals interactions and indirect readout contribute to the discrimination of the degenerate base pair by Bse634I. Structure comparison between related enzymes Bse634I (R/CCGGY), NgoMIV (G/CCGGC) and SgrAI (CR/CCGGYG) reveals how different specificities are achieved within a conserved structural core. Bse634I like other tetrameric REases has two DNA binding interfaces and must synapse two recognition sites to achieve cleavage. It was hypothesised that binding of two recognition sites by tetrameric enzymes contributes to their fidelity. We experimentally determined the fidelity for Bse634I REase in different oligomeric states. Surprisingly, we find that tetramerisation

does not increase Bse634I fidelity in comparison to the dimeric variant. Instead, an inherent ability to act concertedly at two sites provides tetrameric REase with a safety-catch to prevent host DNA cleavage if a single unmodified site becomes available.

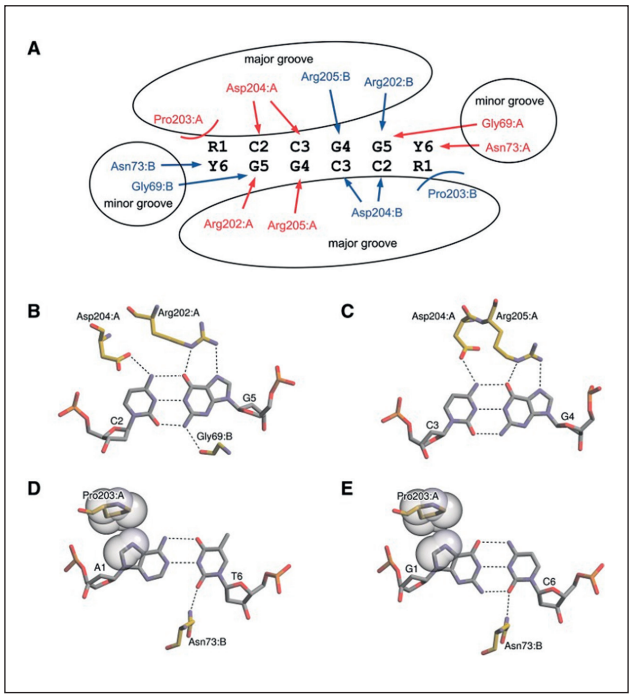

Figure 3. ***Specific interactions of Bse634I with the recognition sequence. (A) Schematic representation of the degenerate target site and interactions between the Bse634I and its target site. Residues belonging to monomers A and B are shown in red and blue, respectively. H-bonds are indicated by arrows, van der Waals interactions to purines–by curved lines. (B) Bse634I interactions with the C2:G5 base pair. (C) Bse634I interactions with the C3:G4 base pair. (D) Bse634I contacts to the outer A1:T6 base pair in the AT-1 complex. (E) Bse634I contacts to the outer G1:C6 base pair in the GC-1 complex. C8 and N7 atoms of R1 base and side chain atoms of Pro203 residue are shown in CPK representation.***

Double challenge: how a monomeric restriction enzyme BcnI recognizes a degenerate DNA sequence and makes a double strand break

Unlike orthodox Type II restriction endonucleases that are homodimers and interact with the palindromic 4-8-bp DNA sequences, BcnI is a monomer which has a single active site but cuts both DNA strands within the 5'-CC↓CGG-3'/3'-GGG↓CC-5' target site ('↓' designates the cleavage position). Therefore, after cutting the first strand, the BcnI monomer must re-bind to the target site in the opposite orientation; but in this case, it runs into a different central base because of the broken symmetry of the recognition site. Crystal-structure analysis shows that to accept both the C:G and G:C base pairs at the center of its target site, BcnI employs two symmetrically positioned histidines H77 and H219 that presumably change their protonation state depending on the binding mode. We show here that a single mutation of BcnI H77 or H219 residues restricts the cleavage activity of the enzyme to either the 5'-CCCGG-3' or the 5'-CCGGG-3' strand, thereby converting BcnI into a strand-specific nicking endonuclease. This is a novel approach for engineering of monomeric restriction enzymes into strand-specific nucleases.

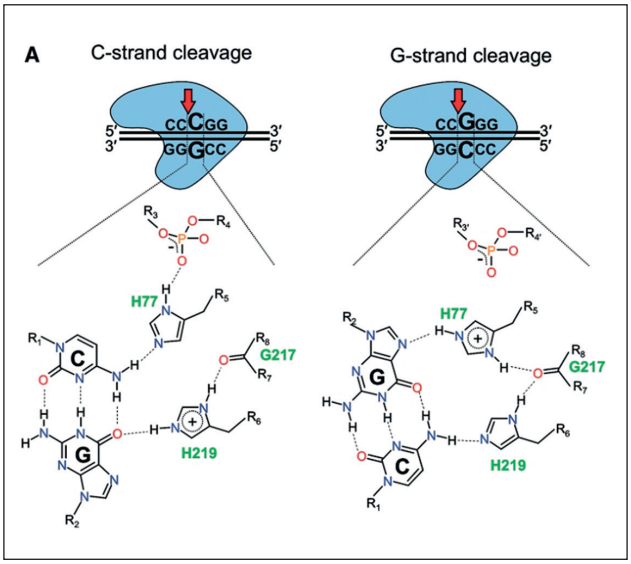

Figure 4. ***Recognition of the central C:G base pair in the BcnI-DNA complex. (A, top) Schematic representation of the wt BcnI interaction with the C-(5'-CCCGG-3') and G-(5'-CCGGG-3') DNA strands. The monomeric enzyme with a single active site cleaves individual DNA strands in two separate nicking reactions. (A, bottom) Major groove interactions with the central base pair in two different enzyme orientations: the C-strand close to the catalytic center [PDB ID: 2ODI] and the G-strand close to the catalytic center (PDB ID: 3IMB). BcnI matches the different pattern of hydrogen bond acceptors and donors in the alternative DNA orientations by switching the protonation state of H219 and H77 residues.***

Endonucleases that generate double-strand breaks in DNA often possess two identical subunits related by rotational symmetry, arranged so that the active sites from each subunit act on opposite DNA strands. In contrast to many endonucleases, Type IIP restriction enzyme BcnI, which recognizes the pseudopalindromic sequence 5'-CCSGG-3' (where S stands for C or G) and cuts both DNA strands after the second C, is a monomer and possesses a single catalytic center. We show that to generate a double-strand break BcnI nicks one DNA strand,

switches its orientation on DNA to match the polarity of the second strand and then cuts the phosphodiester bond on the second DNA strand. Surprisingly, we find that an enzyme flip required for the second DNA strand cleavage occurs without an excursion into bulk solution, as the same BcnI molecule acts processively on both DNA strands. We provide evidence that after cleavage of the first DNA strand, BcnI remains associated with the nicked intermediate and relocates to the opposite strand by a short range diffusive hopping on DNA.

Structure and molecular mechanisms of CRISPR/Cas systems: projects overview

Streptococcus thermophilus DGCC7710 strain, for which biological activity of the CRISPR/Cas system has been directly demonstrated in a phage challenge assay, contains four distinct systems: CRISPR1, CRISPR2, CRISPR3 and CRISPR4, which belong to the three distinct Types. Direct spacer incorporation activity has been demonstrated for the CRISPR1 and CRISPR3 systems, with the former being more active. The CRISPR2 system seems to be disrupted and non-functional, whilst functional activity of CRISPR4 has not yet been demonstrated. Cas genes, which are specific to the repeat regions and fall into different families, are located in close proximity to the spacer-repeat region and encode proteins that often carry functional nucleic-acid related domains such as nucleases, helicases, polymerases and nucleotide binding. We aim to characterize the functional and biochemical activities of Cas proteins belonging to the CRISPR1, CRISPR3 and CRISPR4 systems of *S. thermophilus.*

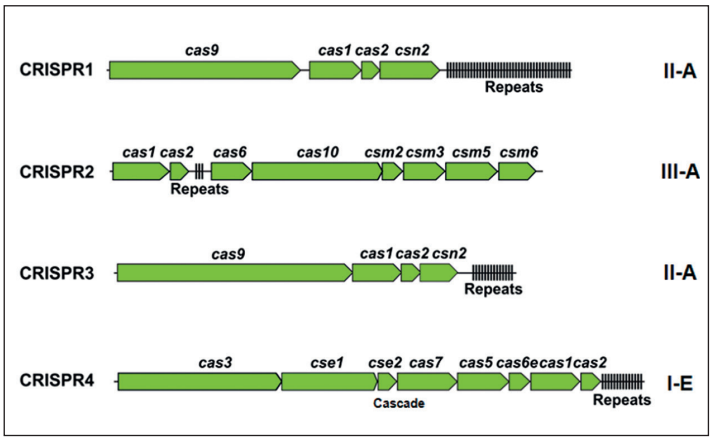

Figure 5. CRISPR/Cas systems of S. thermophilus DGCC7710. CRISPR1 and CRISPR3 systems belong to the TypeII, CRISPR2 to the TypeIII whilst CRISPR4 belongs to the Type I (E.coli subtype).

RNA-guided DNA endonuclease provides DNA silencing in the Type II system

Type II CRISPR-Cas systems typically consist of only four Cas genes. The mechanism for DNA interference provided by the Type II systems remained to be established. We show that in the CRISPR3 system of *Streptococcus thermophilus* (a model and active Type II CRISPR/Cas system), Cas9 associates with crRNA to form an effector complex which specifically cleaves matching target dsDNA. This contrasts sharply with effector complexes for Type I and Type III systems, which are multisubunit ribonucleoprotein complexes. We isolated the Cas9-crRNA complex and demonstrated that it generates *in vitro* a double strand break at specific sites in target DNA molecules that are complementary to crRNA sequences and bear a short proto-spacer adjacent motif (PAM), in the direct vicinity of the matching sequence. We show that DNA cleavage is executed by two distinct active sites (RuvC and HNH) within Cas9, to generate site-specific nicks on opposite DNA strands. Sequence specificity of the Cas9-crRNA complex is dictated by the 42 nt crRNA which includes a 20 nt fragment complementary to the proto-spacer sequence in the target DNA. All together our data demonstrate that the Cas9-crRNA complex functions as an RNA-guided endonuclease with sequence-specific target site recognition and cleavage through two distinct strand nicks.

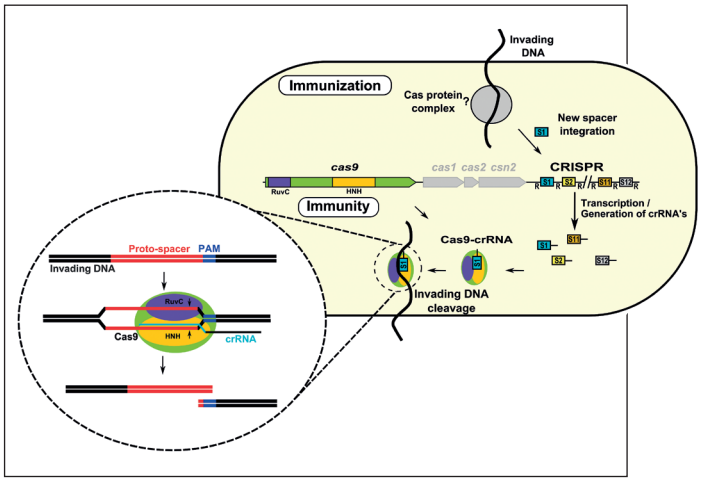

Figure 6. The Cas9-crRNA complex functions as an RNA-guided DNA endonuclease. Guided by the crRNA it finds a specific sequence in the target DNA and Cas9 protein generates two distinct DNA nicks on opposing dsDNA strands that match the loaded small interfering crRNA sequence. Specifically, in the presence of Mg^{2+} ions, the signature Cas9 protein nicks each DNA strands 3 nt -upstream of the PAM sequence to generate blunt DNA ends, through RuvC- and HNH-like active sites that act on separate DNA strands.

This establishes a molecular basis for CRISPR-mediated immunity in bacteria, specifically for Type II systems, which solely rely on the signature Cas9 protein. Further, the simple modular organization of the Cas9-crRNA complex, where specificity for DNA targets is encoded by a small crRNA and the cleavage machinery consists of a single, multidomain Cas protein, provides a versatile platform for the engineering of universal RNA-guided DNA endonucleases. Indeed, by altering the RNA sequence within the Cas9-crRNA complex, programmable endonucleases can be designed both for *in vitro* and *in vivo* applications, and we provide a proof of concept for this novel application. These findings pave the way for the development of novel molecular tools for RNA-directed DNA surgery.

Molecular basis for CRISPR immunity in Type I systems

CRISPR-encoded immunity in Type I systems relies on the Cascade ribonucleoprotein complex, which triggers foreign DNA degradation by an accessory Cas3 protein. It is one of the signature proteins present in the two major types of CRISPR systems. Cas3 contains N-terminal HD-phosphohydrolase and C-terminal SF2 helicase domains. We performed the first experimental characterisation of the Cas3 protein involved in the CRISPR function. We have expressed, purified and functionally characterised the Cas3 protein (St-Cas3) belonging to the CRISPR4 (Ecoli-subtype) system from *Streptococcus thermophilus* DGCC7710. St-Cas3 possesses a single-stranded DNA (ssDNA)-stimulated ATPase activity which is coupled to translocation along ssDNA in 3' to 5' direction. St-Cas3 also shows ATP-independent nuclease activity located in the HD domain with a preference for ssDNA substrates. To dissect the contribution of individual domains, Cas3 separation-of-function mutants (ATPase+, nuclease-) and (ATPase-, nuclease+) have been obtained by site-directed mutagenesis. We suggest that Cas3 ATPase/helicase domain may act as a motor protein which assists delivery of the nuclease activity to Cascade-crRNA complex targeting foreign DNA.

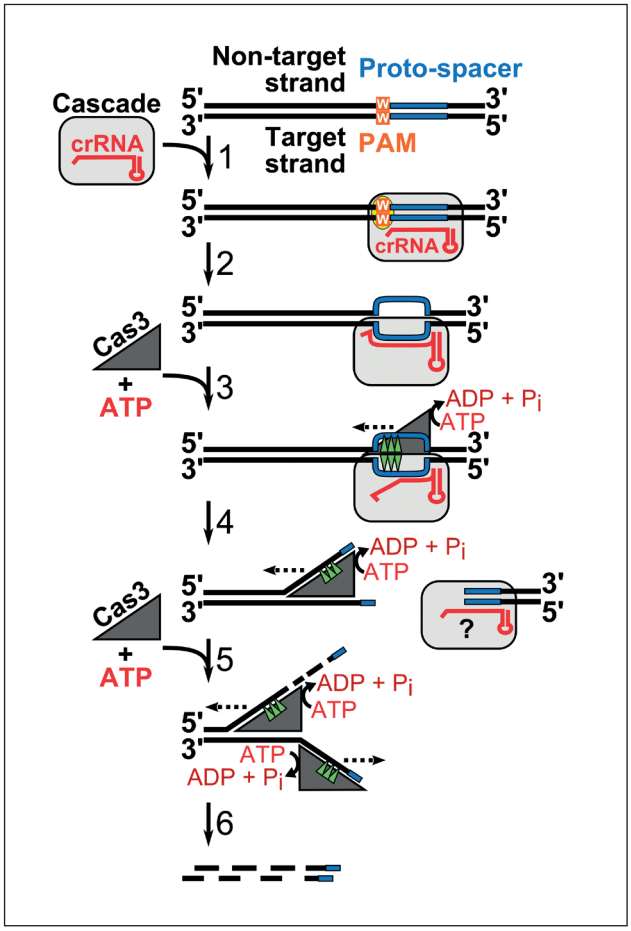

***Figure 7.** Mechanism of DNA interference in the Type I systems. Cascade binding to a matching proto-spacer in the presence of the correct PAM generates an R-loop where the crRNA and the complementary target DNA strand are engaged into a heteroduplex, while the non-target strand is displaced as a single-stranded DNA providing a platform for the Cas3 loading (1). ssDNA binding stimulates the Cas3 ATPase activity which may trigger Cascade remodeling making both DNA strands in the proto-spacer region available for the Cas3 cleavage (2). After cleaving both DNA strands at the proto-spacer Cas3 translocates on the non-target strand in the 3´ to 5´ direction (dashed line) in the ATP-dependent manner and chops the translocating strand using the HD-nuclease domain (3). A stretch of single-stranded DNA created on the complementary strand may promote binding of another Cas3 molecule (4) followed by concomitant cleavage resulting in the degradation of both strands of invading DNA (5).*

To further establish the mechanism for adaptive immunity provided by the *Streptococcus thermophilus* CRISPR4-Cas system (St-CRISPR4-Cas), we isolated an effector complex (St-Cascade) containing 61-nucleotide CRISPR RNA (crRNA). We show that St-Cascade, guided by crRNA, binds *in vitro* to a matching proto-spacer if a proto-spacer adjacent motif (PAM) is present. Surprisingly, the PAM sequence determined from binding analysis is promiscuous and limited to a single nucleotide (A or T) immediately upstream (-1 po-

sition) of the proto-spacer. In the presence of a correct PAM, St-Cascade binding to the target DNA generates an R-loop which serves as a landing site for the Cas3 ATPase/nuclease. We show that Cas3 binding to the displaced strand in the R-loop triggers DNA cleavage, and if ATP is present, Cas3 further degrades DNA in a unidirectional manner. These findings establish a molecular basis for CRISPR immunity in St-CRISPR4-Cas and other Type I systems.

Collaboration

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Dr. Ralf Seidel, Technische Universitat Dresden/Biotech, Dresden, Germany

Funding

EC Framework 7thProgramme
European Social Fund under the Global Grant Measure
Research Council of Lithuania

Publications 2011-2012

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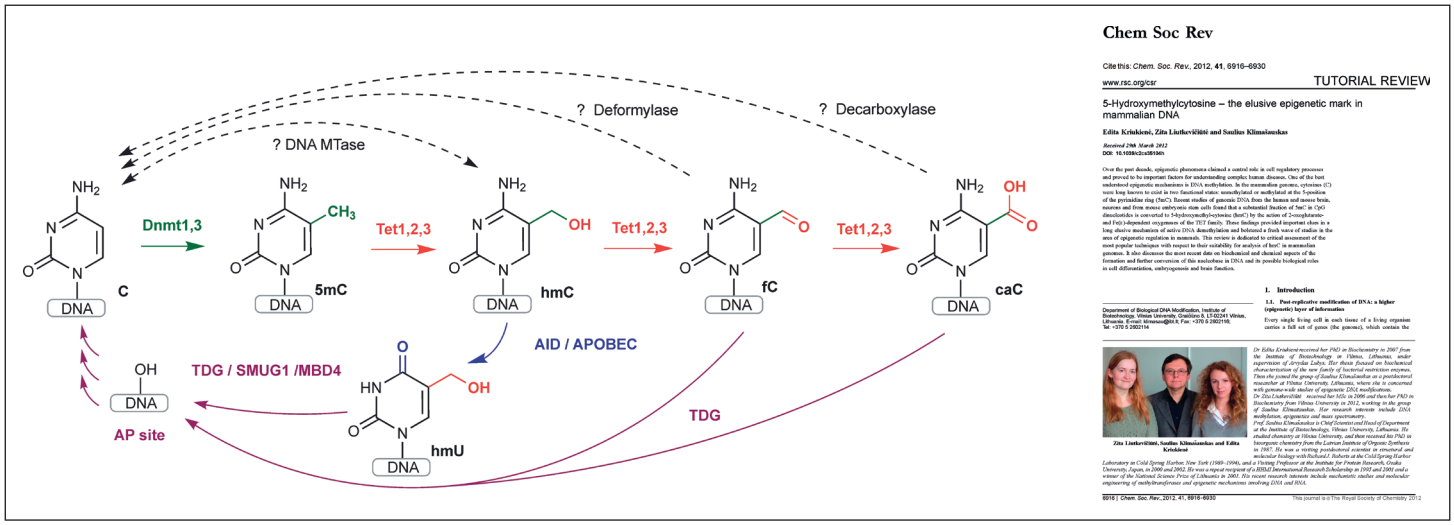
AdoMet-dependent methyltransferases (MTases), which represent more than 3% of the proteins in the cell, catalyze the transfer of the methyl group from S-adenosyl-L-methionine (SAM or AdoMet) to N-, C-, O- or S-nucleophiles in DNA, RNA, proteins or small biomolecules. In DNA of mammals, cytosines are often methylated at the 5-position of the pyrimidine ring to give 5-methylcytosine (5mC). In somatic cells, 5mC is largely restricted to CpG sites. DNA methylation profiles are highly variable across different genetic loci, cells and organisms, and are dependent on tissue, age, sex, diet, and disease. Besides 5mC, certain genomic DNAs have been shown to contain substantial amounts of 5-hydroxymethyl-cytosine (hmC). It was demonstrated that hmC is predominantly produced via oxidation of 5mC residues by TET oxygenases and that the Tet proteins have the capacity to further oxidize hmC forming 5-formylcytosine (fC) and 5-carboxylcytosine (caC) (Fig. 1). Altogether, current evidence suggests that hmC, fC and caC are intermediates on the pathway of active DNA demethylation, and the multiplicity of epigenetic states may also play independent roles in embryonic development, brain function and cancer progression. Therefore, a full appreciation of the biological significance of epigenetic regulation in mammals will require the development of novel tools that allow hmC, 5mC and C to be distinguished unequivocally. We therefore aim to develop new approaches for genome-wide profiling of biological DNA modifications for epigenome studies and improved diagnostics.

Besides their diverse biological roles, DNA MTases are attractive models for studying structural aspects of DNA-protein interaction. Bacterial enzymes recognize an impressive variety (over 300) of short sequences in DNA. As shown first for the HhaI MTase, access to the target base, which is buried within the stacked double helix, is gained in a remarkably elegant manner: by rotating the nucleotide completely out of the DNA helix and into a concave catalytic pocket in the enzyme. This general mechanistic feature named "base-flipping" is shared by numerous other DNA repair and DNA modifying enzymes. Our laboratory has a long standing interest in studies of the mechanistic and structural aspects of DNA methylation using the HhaI DNA cytosine-5 methyltransferase (M.HhaI, recognition target G<u>C</u>GC) from the bacterium Haemophilus haemolyticus as the paradigm model system. The ability of most MTases to catalyze highly specific covalent modifications of biopolymers makes them attractive molecular tools, provided that the transfer of larger chemical entities can be achieved. Our goal is to redesign the enzymatic methyltransferase reactions for targeted covalent deposition of desired functional or reporter groups onto biopolymer molecules such as DNA and RNA.



Chem Soc Rev

Cite this: Chem. Soc. Rev., 2012, 41, 6916–6930

www.rsc.org/csr TUTORIAL REVIEW

5-Hydroxymethylcytosine – the elusive epigenetic mark in mammalian DNA

Edita Kriukienė, Zita Liutkevičiūtė and Saulius Klimašauskas

Received 29th March 2012

DOI: 10.1039/c2cs35076h

Over the past decade, epigenetic phenomena claimed a central role in cell regulatory processes and proved to be important factors for understanding complex human diseases. One of the best understood epigenetic mechanisms is DNA methylation. In the mammalian genome, cytosines (C) were long known to exist in two functional states: unmethylated or methylated at the 5-position of the pyrimidine ring (5mC). Recent studies of genomic DNA from the human and mouse brain, neurons and from mouse embryonic stem cells found that a substantial fraction of 5mC in CpG dinucleotides is converted to 5-hydroxymethyl-cytosine (hmC) by the action of 2-oxoglutarate- and Fe(II)-dependent oxygenases of the TET family. These findings provoked important clues to a long elusive mechanism of active DNA demethylation and bolstered a fresh wave of studies in the area of epigenetic regulation in mammals. This review is dedicated to critical assessment of the most popular techniques with respect to their suitability for analysis of hmC in mammalian genomes. It also discusses the most recent data on biochemical and chemical aspects of the formation and further conversion of this nucleobase in DNA and its possible biological roles in cell differentiation, embryogenesis and brain function.

1. Introduction

1.1. Post-replicative modifications of DNA: a higher (epigenetic) layer of information

Every single living cell in each tissue of a living organism carries a full set of genes (the genome), which contains the

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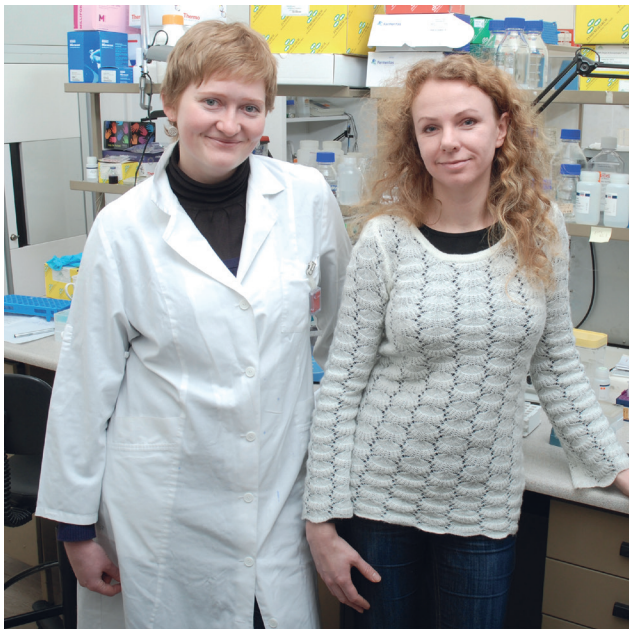
Zita Liutkevičiūtė, Saulius Klimašauskas and Edita Kriukienė

Figure 1. ***Formation and removal of epigenetic marks in mammalian DNA. Cytosine (C) is converted to 5-methylcytosine (5mC) by action of endogenous DNA MTases of Dnmt1 and Dnmt3 families (green pathway). Several mechanisms for DNA demethylation, in which 5-methylcytosine (5mC) is converted back to C, have been proposed. Horizontal arrows represent oxidation-based pathways performed by Tet proteins: methyl group of mC is consecutively oxidized to hydroxymethyl, formyl and carboxyl groups forming 5-hydroxymethylcytosine (hmC), 5-formylcytosine (fC) and 5-carboxycytosine (caC), respectively. Bent plain arrows show deamination-based pathways where hmC is deaminated to 5-hydroxymethyluracil (hmU) in the presence of AID/APOBEC family deaminases, and direct base excision repair (BER) pathways involving TDG, MBD4 and SMUG1 glycosylases, which all lead to transient formation of apyrimidinic (AP) sites in DNA. Dashed arrows denote the newly discovered hydroxymethylation and dehydroxymethylation reactions performed by cytosine-5 methyltransferases in vitro and putative enzymes (deformylase and decarboxylase) which could directly remove the formyl and carboxyl groups from fC and caC, respectively (reviewed in [6]).***

Kinetic and molecular mechanism of DNA methylation

R. Gerasimaitė, E. Merkienė, Z. Staševskij

Enzymatic DNA cytosine-5 methylation is a complex reaction that proceeds via multiple steps such as binding of cofactor AdoMet and substrate DNA, rotation of the target cytosine out of the DNA helix (base flipping), conformational rearrangement of the mobile catalytic loop, activation of the target cytosine via formation of a transient covalent bond and transfer of the methyl group from the bound cofactor onto the target cytosine. We use mutagenesis, biochemical analysis, enzyme kinetics, fluorescence spectroscopy, and x-ray crystalography to delineate the elementary steps on the reaction pathway of the HhaI C5-MTase. Lately, stopped-flow kinetic analysis has been emplyed to directly follow, in a chemically unperturbed system, the target base flipping and its covalent activation. Combined with studies of M.HhaI variants containing redesigned tryptophan fluorophores, we showed that the target base flipping and the closure of the mobile catalytic loop occur simultaneously (Fig. 2). Subsequently, the covalent activation of the target cytosine is closely followed by but is not coincident with the methyl group transfer from the bound cofactor. These findings provide new insights into this physiologically important reaction mechanism and pave the way to in-depth studies of other base-flipping systems [1].



Senior scientists Dr. Zita Liutkevičiūtė and Dr. Edita Kriukienė

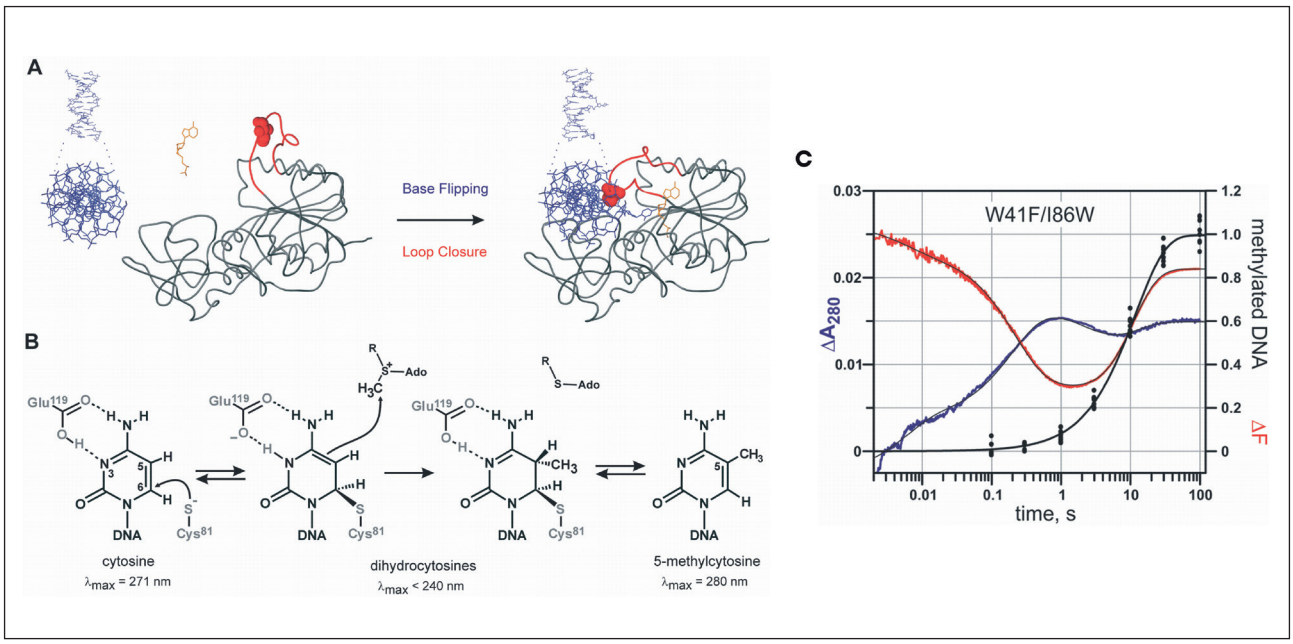


Figure 2. Conformational transitions and covalent catalysis by M.HhaI. (A) M.HhaI flips its target cytosine out of the DNA helix into the active site; the catalytic loop in the protein makes a large motion to lock the target base and the bound AdoMet cofactor. M.HhaI is shown as backbone trace, the catalytic loop (residues 81–100) is red, the engineered Ile86 residue is shown as space fill, DNA and cofactor are represented as sticks models in blue and orange, respectively. (B) The mechanism of covalent target base activation and methyl group transfer by M.HhaI along with associated spectral changes of the target base. (C) Optical and covalent changes observed during catalytic turnover of the W41F/I86W variant of M.HhaI. Stopped-Flow absorbance traces (blue) showing flipping of the target cytosine and formation of the covalent complex, and fluorescence traces showing movements of the catalytic loop (red). The catalytic transfer of methyl groups (black) was measured using a Rapid Quench Flow device [1].

Engineering the catalytic reaction of methyltransferases for targeted covalent labeling of DNA

G. Lukinavičius, V. Masevičius,
G. Urbanavičiūtė, R. Gerasimaitė, M. Tomkuvienė

We had synthesized a series of model AdoMet analogs with sulfonium-bound extended side chains replacing the methyl group and showed that allylic and propargylic side chains can be efficiently transferred by DNA MTases with high sequence- and base-specificity (Dalhoff et al, *Nature Chem. Biol.*, 2006, 2: 31–32; Klimašauskas and Weinhold, *Trends Biotechnol.*, 2007, 25: 99–104) in collaboration with the group of Prof. Elmar Weinhold (RWTH Aachen, Germany). Using DNA MTases along with their novel cofactors that carry useful functional or reporter groups, we demonstrated that our new approach name mTAG (methyltransferase-directed Transfer of Activated Groups) can be used for sequence-specific functionalization and labeling of a wide variety of model and natural DNA substrates (Lukinavicius et al., *J. Amer. Chem. Soc.*, 2007, 129: 2758).

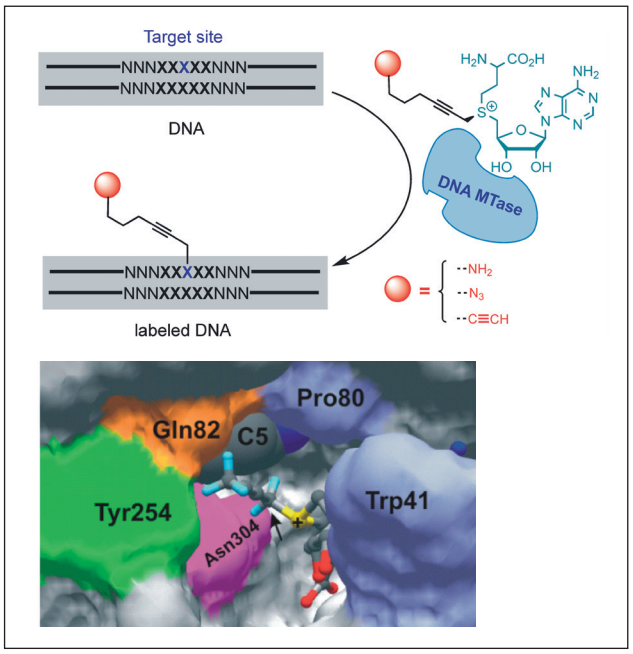


***Figure 3.** Sequence-specific covalent modification of DNA (N and X = nucleotide pairs, XXXXX = recognition sequence of the MTase, X = target nucleotide) catalyzed by a large number of DNA methyltransferases (MTase). Left, methyltransferase-directed Transfer of Activated Groups (mTAG) carrying a reactive functionality (green sphere) from a double-activated AdoMet analog onto a target nucleobase in DNA. Right, Model of the active site of the WT M.HhaI MTase highlights residues Q82, Y254S and N304 whose sidechains occur in close proximity to the extended transferrable group of the bound cofactor analog.*

HPLC-MS analysis performed by senior scientist Dr. V.Masevičius

Although useful in certain applications, our previously synthesized AdoMet analogs carrying sulfonium-bound 4-substituted but-2-ynyl side chains exhibited short lifetimes in physiological buffers. Examination of the reaction kinetics and products showed a fast pH-dependent addition of a water molecule to their transferrable groups. This side reaction was eradicated by synthesis of a new series of analogs, in which the separation between an electronegative group and the triple bond was increased from one to three carbon units. The designed hex-2-ynyl moiety-based cofactor analogs (Fig. 3, left) with terminal amino, azide or alkyne groups showed a markedly improved enzymatic transalkylation activity and proved highly useful for two-step sequence-specific labeling of DNA using engineered DNA C5-MTases [7].

To further optimize the efficacy of the mTAG reactions we performed steric engineering of the cofactor pocket in the M.HhaI C5-MTase by systematic replacement of three non-essential positions, located in two conserved sequence motifs and in a variable region, with smaller residues (Fig. 3, right). We found that double and triple replacements lead to a substantial improvement of the transalkylation activity, permitting competitive mTAG labeling in the presence of AdoMet *in vitro* and in cell lyzates. Analogous replacements of two conserved residues in M.HpaII and M2.Eco31I C5-MTases also resulted in improved transalkylation activity attesting a general applicability of the homology-guided engineering to the C5-MTase family and expanding the repertoire of sequence-specific tools for covalent *in vitro* and *ex vivo* labeling of DNA [3].

Novel non-cofactor reactions of DNA methyltransferases

Z. Liutkevičiūtė, V. Masevičius, I. Grigaitytė, E. Kriukienė

Enzymatic transmethylations generally proceed via a direct nucleophilic attack of a target atom onto the sulfonium-bound methyl group of AdoMet. DNA cytosine-5 MTases use a covalent mechanism for nucleophilic activation of their target cytosine residues. Previously we found that (i) DNA C5-MTases catalyze covalent addition of exogenous aliphatic aldehydes to their target residues in DNA, yielding corresponding 5-*a*-hydroxyalkylcytosines and (ii) can promote the reverse reaction – the removal of formaldehyde from hmC, providing a plausible chemical precedent for an oxidative mechanism of DNA demethylation in mammalian DNA (Liutkevičiūtė et al., *Nat. Chem. Biol.,* 2009, 5: 400–402). Most intriguingly, we have recently discovered that bacterial C5-MTases can catalyze *in vitro* the condensation of aliphatic thiols and selenols to 5-hydroxymethylcytosine in DNA yielding 5-chalcogenomethyl derivatives (Fig. 4). These new atypical reactions open new ways for sequence-specific derivatization and analysis of 5-hydroxymethylcytosine, a recently discovered nucleobase in mammalian DNA (see Fig. 1 and [6]).

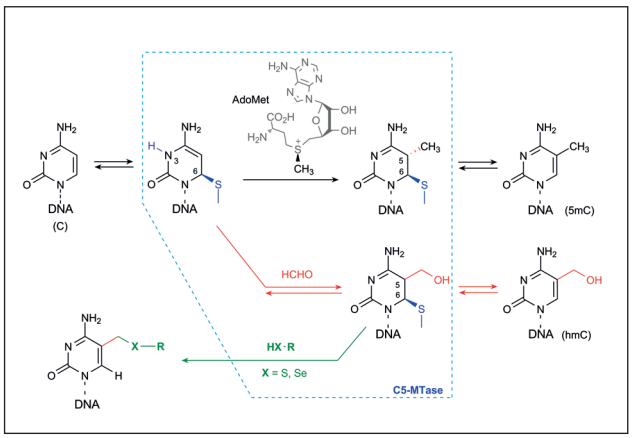


Figure 4. ***Catalytic versatility of DNA C5-MTases. DNA C5-MTases use covalent catalysis for the transfer of the methyl group from the cofactor AdoMet on their target cytosine residue (upper reaction) yielding 5-methylcytosine (5mC). In the absence of AdoMet, C5-MTases can catalyze nucleophilic addition of exogenous aldehydes (red) to the target cytosine to give corresponding 5-(α-hydroxyalkyl)cytosines (shown is reaction with formaldehyde yielding 5-hydroxymethylcytosine, hmC) in vitro. This modification is reversed back to unmodified DNA by the enzyme in the absence of the exogenous aldehyde. C5-MTases can also catalyze nucleophilic addition of exogenous aliphatic thiols and selenols to 5-hydroxymethylcytosine in DNA (green) yielding 5-chalcogenomethyl derivatives [2]. The thiol group from a catalytic cysteine residue of the C5-MTase is shown in blue.***

Methylation of small RNAs

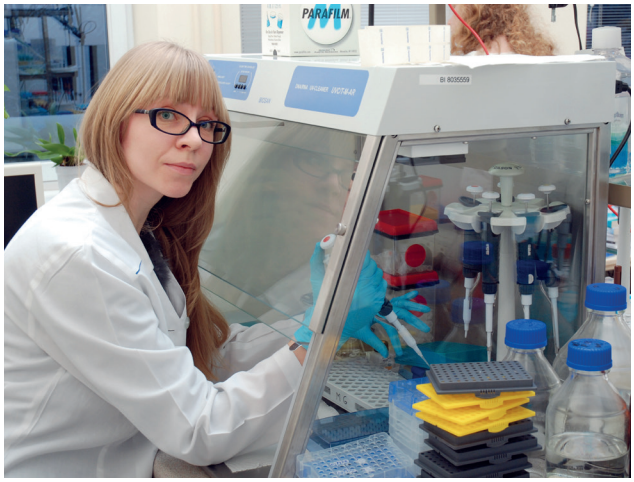
G. Vilkaitis, A. Plotnikova, S. Baranauskė, A.Osipenko, S. Butkytė

There is a growing recognition that cells of all living organisms produce many thousands of short non-coding RNAs. Functionally important small RNAs such as miRNAs, siRNAs and piRNAs are essential for post-transcriptional gene regulation in eukaryotic organisms including humans. Biogenesis of plant miRNAs and siRNAs or animal piRNAs and Ago2-loaded siRNAs involves modification of small RNA molecules at the 2'-O group of 3'-termini. The paradigm of the small RNA 2'-O-methyltransferases family - large multidomain methyltransferase HEN1 from *Arabidopsis thaliana* has been implicated in biogenesis pathways and is the only invariable protein in biogenesis of all small RNA types in plants (miRNA, ta-siRNA, nat-siRNA, ls-siRNA, hc-siRNA *etc*). Unlike tRNA or rRNA methylases, HEN1 modifies two ends of a short duplex in succession and thus represents a unique class of RNA enzymes interacting with pseudo-symmetric substrates. Our studies indicate that HEN1 specifically binds double-stranded unmethylated or hemimethylated miR173/miR173* substrates with sub-nanomolar affinity in a cofactor-dependent manner. The N-terminal domain is a key factor for the tight RNA binding, however stabilization of HEN1•miRNA/miRNA* complex is under strict control of the catalytic domain. Kinetic studies under single turnover and pre-steady state conditions in combination with isotope partitioning analysis showed that binary HEN1•miRNA/miRNA* complexes are catalytically competent and successive methylation of the two strands in a RNA duplex occurs in a non-processive (distributive) manner. We also find that a moderate methylation strand preference is exerted at the binding step but is not determined by interactions with the 3'-terminal nucleotide. The obtained results provide valuable insights into the enzymatic mechanism of 3'-terminal 2'-O-methylation and biological role of an abundant class of RNA 2'-O-methyltransferases, which share similar catalytic domains, and are widely distributed in all biological kingdoms except archea. Since the mode of interaction and modification of double-stranded RNA resembles interactions of monomeric DNA enzymes (such methyltransferases) with their palindromic target sites, our studies thus establish a mechanistic paradigm for terminal modifications of nucleic acids duplexes [8].

Archaeal C/D box RNPs: synthetically programmable sequence-specific labeling of RNA

M. Tomkuvienė, G. Lukinavičius, I. Černiauskas

In archaea, C/D box small ribonucleoprotein complexes (sRNPs) direct site specific 2'-O methylation to numerous important sites in ribosomal and transfer RNA. The sRNPs are comprised of a C/D guide RNA which binds two sets of core proteins L7Ae, Nop5p, and aFib (the methyltransferase). Base pairing of a guide sequence in the guide RNA to the RNA substrate targets the modifying enzyme to the site of methylation. In collaboration with Dr. Beatrice Clouet d'Orval at Université Paul Sabatier, we have *in vitro* reconstituted a functional box C/D small ribonucleoprotein RNA 2'-O-methyltransferase (C/D RNP) from the thermophilic archaeon *Pyrococcus abyssi*



RNA labeling experiments performed by junior scientist M.Tomkuvienė

and demonstrated its ability to transfer a propynyl group from a selenium-based synthetic cofactor analog (kindly provided by Prof. E. Weinhold, RWTH Aachen, Germany) to a series of preselected target sites in model tRNA and pre-mRNA molecules. Target selection of the RNP was programmed by changing a dodecanucleotide guide sequence in the 64-nt C/D guide RNA leading to efficient derivatization of three out of four new targets in each RNA substrate. We also showed that the transferred terminal alkyne can be further appended with a fluorophore using a bioorthogonal Huisgen 1,3-cycloaddition (click) reaction (Fig. 5). The described approach for the first time permits synthetically tunable sequence-specific labeling of RNA with single-nucleotide precision [4].

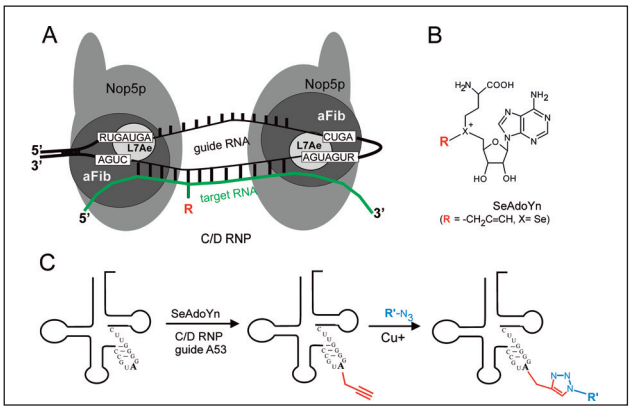


***Figure 5.** Programmable sequence-specific labeling of RNA [4]. (A) Schematic structure of a C/D RNP complex with substrate RNA. C/D RNP comprises two sets of core proteins L7Ae, Nop5p and aFib assembled around two regions of conserved sequences (called C/D and C'/D' sites) of a guide RNA. One of the variable guide sequences is shown base-paired to a target sequence of the substrate RNA (green), in which a defined nucleotide is modified with an extended group R transferred from a cofactor analog; (B) Structure of the selenium-based AdoMet analog SeAdoYn; (C) Two-step covalent labeling of tRNA-Leu via a C/D A53 RNP-directed propynylation (red), followed by chemo-selective coupling of a fluorogenic azide derivative (blue).*

Molecular tools for epigenome profiling

E. Kriukienė, Z. Liutkevičiūtė, G. Urbanavičiūtė, A. Lapinaitė, Z. Staševskij

We aim to develop new experimental approaches to genome-wide profiling of DNA modifications for epigenome studies and improved diagnostics. One such strategy is based on selective mTAG labeling and enrichment of unmethylated CpG sites in the genome followed by analysis of the enriched fractions on tiling microarrays (in collaboration with Prof. Art Petronis, CAMH, Canada) [9]. A substantial effort is also dedicated to the development of new technologies that are capable of mapping of hmC in mammalian genomes based on known and the newly discovered enzymatic transformations of the hydroxymethyl group in hmC [2]. Combining these novel approaches with DNA microarray analyses and next generation sequencing, we started studies of epigenome-wide distribution of the modified cytosines to unveil the intra- and inter- individual variation of modification patterns in disease-related human DNA samples. One of the most intriguing peculiarities of hmC is its high abundance in brain tissues. In a multilateral collaborative study we characterized the genomic distribution of hmC and 5mC in human and mouse tissues. We

assayed hmC by using glucosylation coupled with restriction-enzyme digestion and microarray analysis. We detected hmC enrichment in genes with synapse-related functions in both human and mouse brain. We also identified substantial tissue-specific differential distributions of these DNA modifications at the exon-intron boundary in human and mouse. This boundary change was mainly due to hmC in the brain (Fig. 6) but due to 5mC in non-neural contexts. This pattern was replicated in multiple independent data sets and with single-molecule sequencing. Moreover, in human frontal cortex, constitutive exons contained higher levels of hmC relative to alternatively spliced exons. Our study suggests a new role for hmC in RNA splicing and synaptic function in the brain [5].

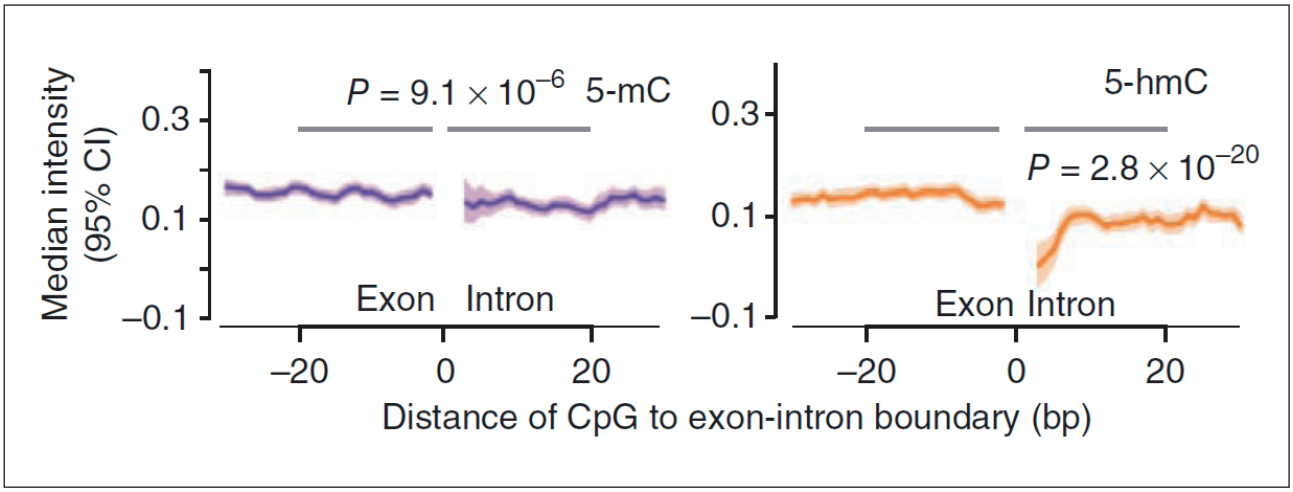


Figure 6. 5-Hydroxymethylcytosine marks exon-intron boundaries in the human brain. DNA microarray probe intensities for 5-mC (left) and 5-hmC (right) as a function of distance (d) from the boundary (28 brain samples, 6 chromosomes); x axis, distance (bp) of modifiable cytosine from boundary; y axis, median intensity of sample-averaged probes (5-bp-centered smoothing). P values are from linear mixed-effects model (exon versus intron, d ≤ 20 bp from boundary); shading shows 95% CI [5].

Collaboration

Prof. Art Petronis, CAMH, Toronto U., Canada
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Funding

National Institutes of Health/National Human Genome Research Institute (USA)
European Social Fund under the Global Grant measure
EC Framework 7th Programme
Research Council of Lithuania
Bilateral French-Lithuanian Collaborative Programme 'Gilibert'
COST

Research Contracts

Thermo Fisher Scientific, Lithuania

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* Featured article

** VIP - very important paper

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Human metapneumovirus

Human metapneumovirus (hMPV), first isolated in the Netherlands in 2001, is a member of the *Paramyxoviridea* family. It has been tentatively assigned to the *Metapneumovirus* genus of the *Pneumovirus* subfamily (van den Hoogen et al., 2001). Since its discovery, hMPV has been found to infect humans worldwide. HMPV has been recognized as a common cause of respiratory infections, ranging from upper respiratory tract infections to severe lower respiratory tract infections in very young children, elderly individuals and immunocompromised patients. Similar to other members of *Paramyxoviridea* family, hMPV is an enveloped single-stranded negative-sense RNA virus. Its genome is approximately 13 kb in length and contains nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), attachment (G) and RNA-dependent RNA polymerase (L) genes in the order 3'-N-P-M-F-M2-SH-G-L-5'. Extensive sequence analysis of virus isolates from around the world indicates that there are two major genetic lineages of hMPV. With the notable exception of the SH and G proteins, the polypeptides encoded by each of these genetic lineages are highly related at the amino acid (aa) level. For example, the aa sequence identity of the predicted encoded proteins between these two groups of hMPV strains is 96% for the N protein. The diagnosis of hMPV infection usually requires the detection of viral RNA in clinical specimens, although serological assays using isolated viral antigens or direct antibody-based detection of the virus in respiratory secretions may provide new possibilities for laboratory diagnosis of hMPV infection and wide epidemiological studies. Virus recombinant proteins are promising tools for development of vaccines and diagnostics. The aim of the current study was to construct an efficient high-level yeast expression system for the generation of hMPV nucleocapsid (N) protein and to develop monoclonal antibodies (MAbs) suitable for hMPV detection. The genome of hMPV was isolated from oral fluid of an infected patient by using specific primers and reverse transcriptase polymerase chain reaction (RT-PCR). DNA sequence corresponding to the N protein gene was inserted into yeast expression vector under inducible *GAL7* promoter. SDS-PAGE analysis of crude lysates of yeast *S. cerevisiae* harboring recombinant plasmid revealed the presence of a protein band of approximately 43 kDa corresponding to the molecular weight of hMPV N protein. Electron microscopy analysis of purified N protein revealed nucleocapsid-like structures with typical herring-bone morphology: rods of 20 nm diameter with repeated serration along the edges and central core of 5 nm. Recombinant hMPV N protein was reactive with human serum specimens collected from patients with confirmed hMPV infection. After immunization of mice with recombinant hMPV N protein, a panel of MAbs was generated. The specificity of newly generated MAbs was proven by immunofluorescence analysis of hMPV-infected cells. Epitope mapping using truncated variants of hMPV N revealed localization of linear MAb epitopes at the N-terminus of hMPV N protein, between amino acid residues 1 and 90. The MAbs directed against conformational epitopes did not recognize hMPV N protein variants containing either N- or C-terminal truncations. The reactivity of recombinant hMPV N protein with hMPV-positive serum specimens and the ability of MAbs to recognize virus-infected cells confirm the antigenic similarity between yeast-expressed hMPV N protein and native viral nucleocapsids. In conclusion, recombinant hMPV N protein and hMPV-specific MAbs provide new diagnostic reagents for hMTP infection.

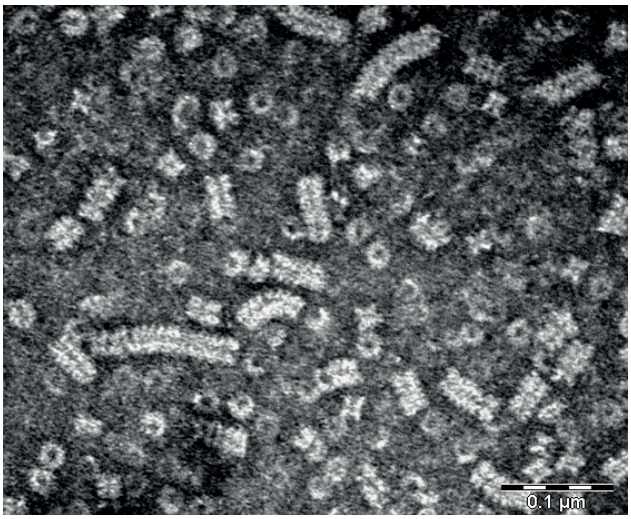


Figure 1. ***Yeast derived hMPV nucleocapsid protein. Scale bar 100nm.***

Human bocavirus

Human bocavirus (HBoV1), first described in 2005, was considered a causative agent of previously unexplained respiratory tract diseases. Recently, 3 new members of genus *Bocavirus,* HBoV2-4 were described. HBoV2-4 occurs mainly in the gastrointestinal tract but rarely in the respiratory tract, contrary to HBoV1. Recombinant viral antigens have been proven useful for serologic diagnosis of viral infections. Production of

HBoV1-4 antigens in yeast expression system has not yet been reported. , In the current study, the capsid proteins VP1 and VP2 of HBoV1were expressed in yeast *S.cerevisiae*. Electron microscopy demonstrated that both purified recombinant proteins self assembled into virus-like particles (VLPs) exhibiting the typical icosahedral appearance of parvoviruses with a diameter of approximately 20 nm. The yield and stability of HBoV1 VP2 protein was significantly higher in comparison to VP1, therefore VP2 was chosen for the developing of an immunoassay and generation of monoclonal antibodies. HBoV1 VP2 VLPs were stable in yeast and were easily purified by caesium chloride gradient ultracentrifugation. Therefore, yeast expression system proved to be simple, efficient and cost-effective, suitable for high-level production of HBoV1 VP2 as VLPs. Four monoclonal antibodies (MAbs) of IgG1 subtype were generated by immunization of mouse with recombinant HBoV1 VP2 VLPs. Three of them specifically recognized only HBoV1 VP2 protein; one MAb was cross-reactive with HBoV2 and HBoV4 VP2 proteins. Recombinant HBoV1 VP2 VLPs and VP2-specific MAbs were employed to develop serological assays to detect virus-specific IgG antibodies in human serum specimens.

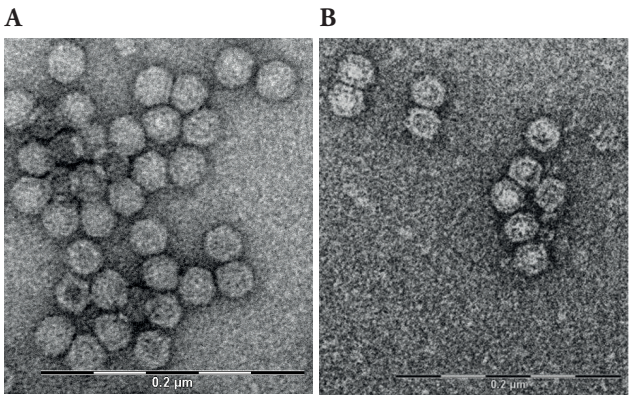
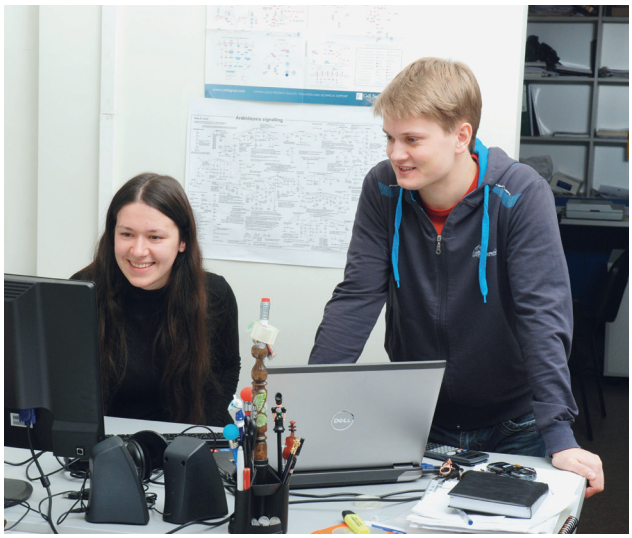


***Figure 2.** Electron micrograph of CsCl purified VLPs. Scale bar 200nm.* **A** *- HBoV1 VP2*; **B** *- HBoV1.*

Human PARV4

Human parvovirus 4 (PARV4) is a recently discovered new member of the *Parvoviridea* family not closely related to any of the known human parvoviruses. PARV4 is widely distributed in intravenous drug users, particularly in those co-infected with human immunodeficiency virus and hepatitis C. PARV4 has been isolated from plasma of individuals with symptoms of acute viral infection, however, till now PARV4 has not been associated with any disease and its prevalence in human population is not yet clearly established. In the current study, the major capsid protein VP2 of PARV4 was generated in yeast *Sacharomyces cerevisiae* and used for serological detection of virus-specific IgG and IgM in the sera of low risk individuals. One-hundred seventy serum specimens obtained from patients with acute respiratory diseases were tested for PARV4-specific IgG and IgM antibodies. Sixteen (9.4%) seropositive individuals were diagnosed, including 10 IgG positive, 12 IgM positive and 6 both IgG and IgM positive. Six of 16 sero-positive



PhD student Kotryna Kvederavičiūtė and bioengineer Justinas Šimulis

MAb clone	Optical density (OD450) values by an indirect ELISA					
	HBoV1 VP2	HBoV2 VP2	HBoV3 VP2	HBoV4 VP2	B19	BPV1
4C2	3.7260	0.0105	0.0320	0.0160	0.0055	0.0085
9G12	1.0770	0.0085	0.0040	0.0115	0.0025	0.0055
12C1	3.0281	0.0106	0.0076	0.0121	0.0051	0.0036
15C6	3.3491	3.4706	0.0166	3.0376	0.0071	0.0081

***Table.** The cross-reactivity of MAbs rose against yeast-expressed HBoV1 VP2 VLPs with recombinant VP2 proteins of related human parvoviruses.*

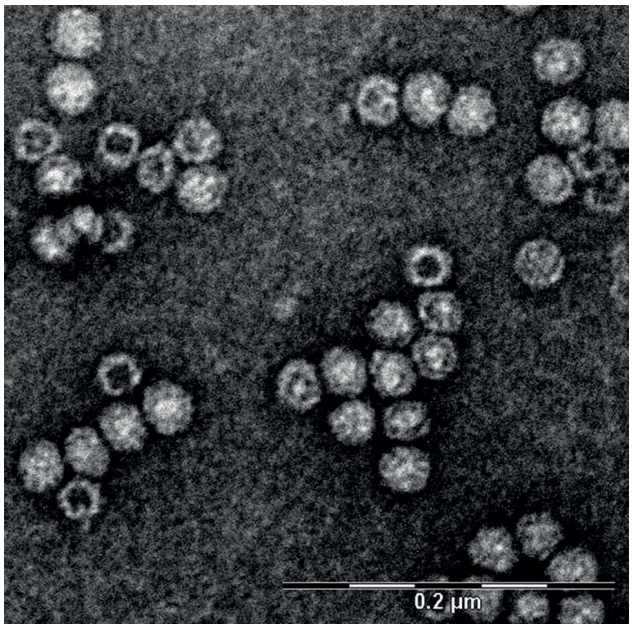


Figure 3. Electron micrograph of CsCl purified PARV4 VP2 VLPs. Scale bar 200 nm.

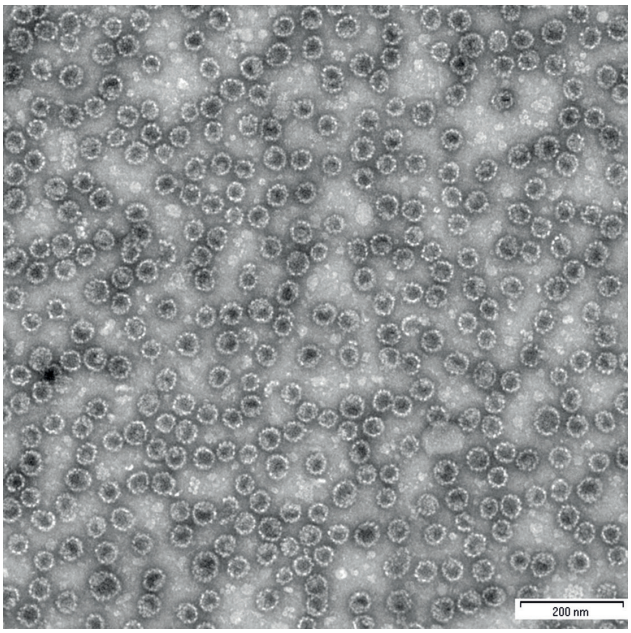


Figure 4. Electron micrograph of purified VLPs of polyomavirus VP1/VP2 proteins. Scale bar 200 nm.

individuals were 3-11 years old children. None of them had an evidence of parenteral exposure to PARV4 infection. Our data demonstrate that recombinant yeast-derived VP2 protein self-assembled to virus-like particles represent a useful tool for studying the seroprevalence of PARV4 infection. The presence of PARV4-specific antibodies in a low-risk group may indicate the possibility of alternative routes of virus transmission.

Hamster polyomavirus

Expression system in yeast for polyomavirus virus like particles (VLPs) formed from VP1 or VP1 and VP2 proteins has been developed. Recombinant VLPs derived from most of known polyomaviruses strains were generated in yeast and used for various applications. Hamster polymavirus (HaPyV) major capsid protein VP1-based VLPs appeared as powerful vehicles for the presentation of foreign antigens. VP1-derived VLPs tolerated inserts of different size and origin at certain VP1 sites. Inserted peptides were exposed on surface of VLPs and these VLPs were successfully used for production of monoclonal antibodies (US Patent No.:US7,919,314 B2. Apr. 5, 2011). Using model GP33 CTL epitope derived from murine Lymphocytic choriomeningitis virus inserted into HaPyV VP1 protein it was shown that chimeric VP1-GP33 VLPs were efficiently processed in antigen presenting cells *in vitro* and *in vivo* and were capable to induce antigen-specific CD8+ T cell proliferation and CTL response in mice. Our latest studies have showed that HaPyV-derived VLPs provide even more possibilities for protein engineering. The exploitation of VP2 protein fused with large (370-472 aa long) recombinant antibody molecule as a subunit of VLPs represents the new way to obtain correctly folded and functionally active complex proteins expressed on VLPs surface formed of pseudotype VP1/VP2 proteins.

Analysis of stress responses in yeast

Expression of recombinant proteins is connected to various stress responses in yeast cells. For example, overexpression of human virus surface protein precursors in yeast cytoplasm induces the UPR's (unfolded protein response) cytosolic counterpart, the UPR-Cyto, which represent a subset of proteins involved in the heat-shock response. We are studying this phenomenon both by using analysis of involved cellular proteins and processes at the molecular level and by evaluation of induced morphological alterations in yeast cells *in vivo*. The example of observed morphological alterations during the UPR-Cyto stress in yeast *S.cerevisiae* is shown in Fig. 5. The reasons for such dramatic changes in cellular morphology are revealed by proteomic studies of differentially expressed proteins in

UPR-Cyto and analysis of their interactions with the recombinant proteins (for more information see [Čiplys et al., 2011, *Microb Cell Fact.* 10:37.]).

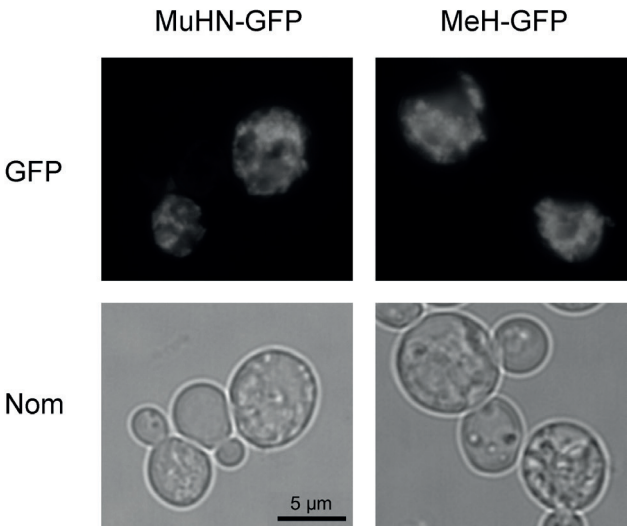


Figure 5. ***Yeast cells overexpressing viral proteins.***

Microscopic view of *S.cerevisiae* cells overexpressing green fluorescent protein (GFP) fusions to mumps hemagglutinin-neuraminidase (MuHN) and measles hemagglutinin (MeH) proteins that induce the UPR-Cyto stress in yeast. Cells that overexpress these proteins are visualized by GFP fluorescence analysis (top panel). They reveal dramatic morphological alterations (Nomarski images of the same cells in the bottom panel). For comparison neighbouring cells, non-expressing these proteins exhibit normal intracellular morphology.

Manipulation of cell culture conditions to improve recombinant protein yield in yeast

It is well known that recombinant protein expression level depends on cell culture conditions. We are studying these effects in more detail, focusing on the growth phases of yeast cultures. For such experiments, it is important to determine the exact growth curves of yeast transformants used for the expression studies. An example of *S.cerevisiae* strain growth curve is shown in Fig. 6. In the expression experiments, usually the culture is subjected to defined changes of conditions (e.g. is shifted to different temperature) at different growth phases and the yields of recombinant protein are compared at the end of cultivation.

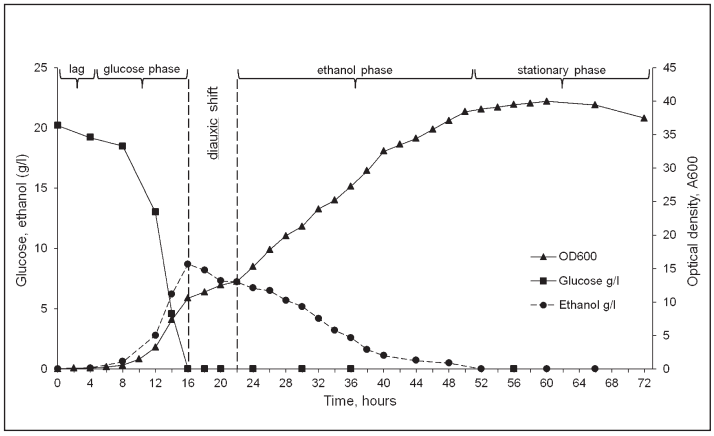


Figure 6. ***Growth phases of yeast cells.***

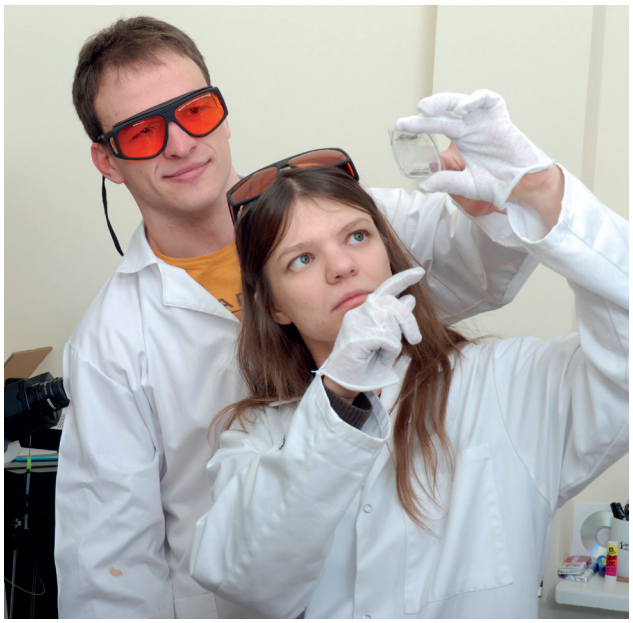
Growth phases of *S.cerevisiae* AH22 strain transformant grown in YEPD medium. Triangles indicate culture optical density, squares denote glucose and the circles – ethanol concentrations in the medium at the time intervals plotted on X axis. The growth phases are indicated at the top.

Development of novel antiviral drugs against Influenza

Overall success of the Fluinhibit project (FP7 Health - 2007-2.3.3.7: supporting highly innovative inter-disciplinary research on influenza, grant No. 201634, 2008-2010) let us to extent our investigation in the collaborative FP7 project Flucure (FP7-Influenza-2010, grant No. 259972, 2010-2014) aimed to discover the inhibitors of influenza viral RNA polymerase and prepare new platform for development of antiviral drugs based on viral polymerase inhibition. RNA polymerase subunits PA, PB1 and PB2, have been successfully expressed in yeast *Sacharomyces cerevisiae* and *Pichia pastoris* and purified. Yeast expressed PA/PB1 dimer was also purified to more than 90% purity as well as different PA and PB1 fuse variants with luciferase used for detection of PA/PB1 complex. Purified subunits and fused proteins of influenza polymerase are provided to the project partners for further applications in selection and analysis of influenza virus inhibitors.

Anthocyanin research

The projects „Interspecies hybrids of horticultural plants as the new source of anthocyanins“ and „Expression of anthocyanin



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synthesis pathway genes in horticultural plants" aims to elucidate anthocyanin synthesis pathways in plants of *Fragaria* and *Prunus* genera belonging to *Rosaceae* family and *Ribes* genus belonging to *Saxifragales* order. MYB and bHLH transcription factors are the key players responsible for pigmentation differences in separate varieties or species of the same genera. The sequences of these genes from several *Fragaria*, *Prunus* and *Ribes* species are cloned and are currently under analysis. The detailed expression studies in horticultural plants along with functional studies in transient expression systems are undertaken. Obtained information will be used by horticultural specialists for directional breeding of *Fragaria*, *Prunus* and *Ribes* varieties with high anthocyanin content.

Effect of elevated ozone concentration on host-viroid interaction

It is known that plant susceptibility to fungal, bacterial and viral plant pathogens may be significantly altered by elevated concentration of tropospheric ozone. However the effect of elevated ozone concentration on pathogenicity and spread of the subviral pathogen remains unknown. Our studies are devoted to get some evidence if plant and sub-viral pathogen interaction may be altered by the elevated ozone concentration. The wide range of ozone treatments was applied on the experimental system, formed from the tomato variety Micro-Tom and potato spindle tuber viroid. Significant of differences in plant response to elevated ozone concentration between inoculated and not inoculated plants were strongly influenced by ozone treatment indicating that pathogenicity of potato spindle tuber viroid can be altered by the exposure of inoculated plants to severe ozone stress. (Fig. 7).

Differences in the plant height and the degree of defoliation observed between viroid infected (in the left) and uninfected (in the right) tomatoes (*lycopersicon esculentum* Mill.) cv. "Micro-Tom" in six weeks after acute ozone treatment (400ppb x 6h) carried on young plants.

***Figure 7.** Viroid infection in tomatoes.*

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Funding

EC Framework 7th Programme
Research Council of Lithuania

Research Contracts

Microimmune Ltd., London, U.K.
Euroimmune AG, Germany
UAB Profarma, Lithuania
DiaSorin S.p.A.
Abcam Ltd., U.K.

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Department of Immunology and Cell Biology consists of three research groups. In 2011-2012, the research was focussed to the following topics: development of monoclonal and recombinant antibodies; studies on bacterial virulence factors (Dr. A.Žvirblienė), regulation of gene expression by alternative splicing (Dr. A.Kanopka), molecular epidemiology of tuberculosis (Dr. P.Stakėnas).

Generation of recombinant virus-like particles harboring functionally active antibody fragments

Recombinant antibodies can be produced in different formats and different expression systems. Single chain variable fragments (scFvs) represent an attractive alternative to full-length antibodies and they can be easily produced in bacteria or yeast. However, the scFvs exhibit monovalent antigen-binding properties and short serum half-lives. The stability and avidity of the scFvs can be improved by their multimerization or fusion with IgG Fc domain. The aim of the current study was to investigate the possibilities to produce in yeast high-affinity scFv-Fc proteins neutralizing the cytolytic activity of vaginolysin (VLY), the main virulence factor of *Gardnerella vaginalis*.

The scFv protein derived from hybridoma cell line producing high-affinity neutralizing antibodies against VLY was fused with human IgG1 Fc domain. Four different variants of anti-VLY scFv-Fc fusion proteins were constructed and produced in yeast *Saccharomyces cerevisiae*. The non-tagged scFv-Fc and hexahistidine-tagged scFv-Fc proteins were found predominantly as insoluble aggregates and therefore were not suitable for further purification and activity testing. The addition of yeast α-factor signal sequence did not support secretion of anti-VLY scFv-Fc but increased the amount of its intracellular soluble form. However, the purified protein showed a weak VLY-neutralizing capability. In contrast, the fusion of anti-VLY scFv-Fc molecules with hamster polyomavirus -derived VP2 protein and its co-expression with VP1 protein resulted in an effective production of pseudotype virus-like particles (VLPs) that exhibited a strong VLY-binding activity. Recombinant scFv-Fc molecules displayed on the surface of VLPs neutralized VLY-mediated lysis of human erythrocytes and HeLa cells with high potency comparable to that of full-length antibody.

In conclusion, the new approach to display the scFv-Fc molecules on the surface of pseudotype VLPs was successful and allowed generation of multivalent scFv-Fc proteins with high VLY-neutralizing potency. Our study demonstrated for the first time that large recombinant antibody molecule fused with hamster polyomavirus VP2 protein and co-expressed with VP1 protein in the form of pseudotype VLPs was properly folded and exhibited strong antigen-binding activity.

The current study broadens the potential of recombinant VLPs as a highly efficient carrier for functionally active complex proteins.

This work was performed in collaboration with the Department of Eukaryote Gene Engineering.

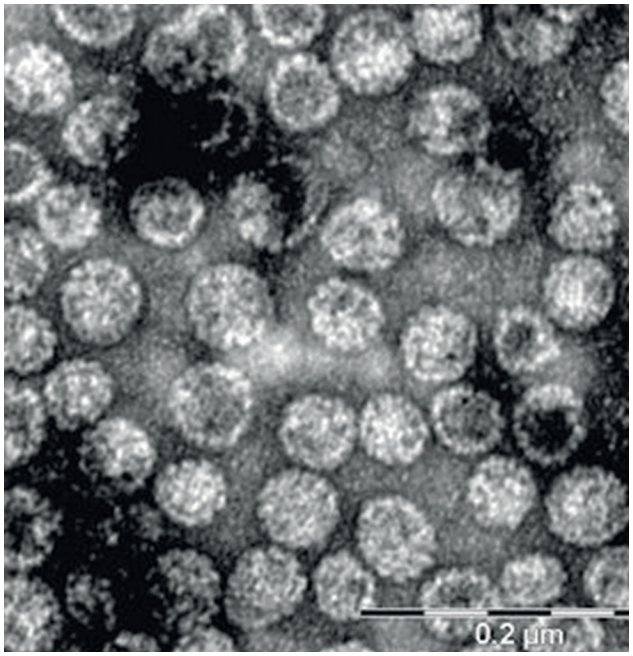


***Figure. 1.** Electron microscopy pictures of VP1/VP2-scFv-Fv pseudotype VLPs, stained with 2% aqueous uranyl acetate solution and examined by Morgagni 268 electron microscope.*

Reference: Pleckaityte et al., *Microb. Cell Fact.* 2011 10:109.

Studies on genetic and biochemical diversity of *Gardnerella vaginalis* strains

Gardnerella vaginalis is considered a substantial player in the progression of bacterial vaginosis (BV). We analysed 17 G. vaginalis strains isolated from the genital tract of women diagnosed with BV to establish a potential link between genotypes/biotypes and the expression of virulence factors, vaginolysin (VLY) and sialidase, which are assumed to play a substantial role in the pathogenesis of BV. Amplified ribosomal DNA restriction analysis revealed two G. vaginalis genotypes. Gardnerella vaginalis isolates of genotype 2 appeared more complex than genotype 1 and were subdivided into three subtypes. Biochemical typing allowed us to distinguish four different biotypes. A great diversity of the level of VLY production among the isolates of G. vaginalis may be related to a different cytotoxicity level of

the strains. We did not find any correlation between VLY production level and G. vaginalis genotype/biotype. In contrast, a link between G. vaginalis genotype and sialidase production was established. Our findings on the diversity of VLY expression level in different clinical isolates and linking sialidase activity with the genotype of G. vaginalis could help to evaluate the pathogenic potential of different G. vaginalis strains.

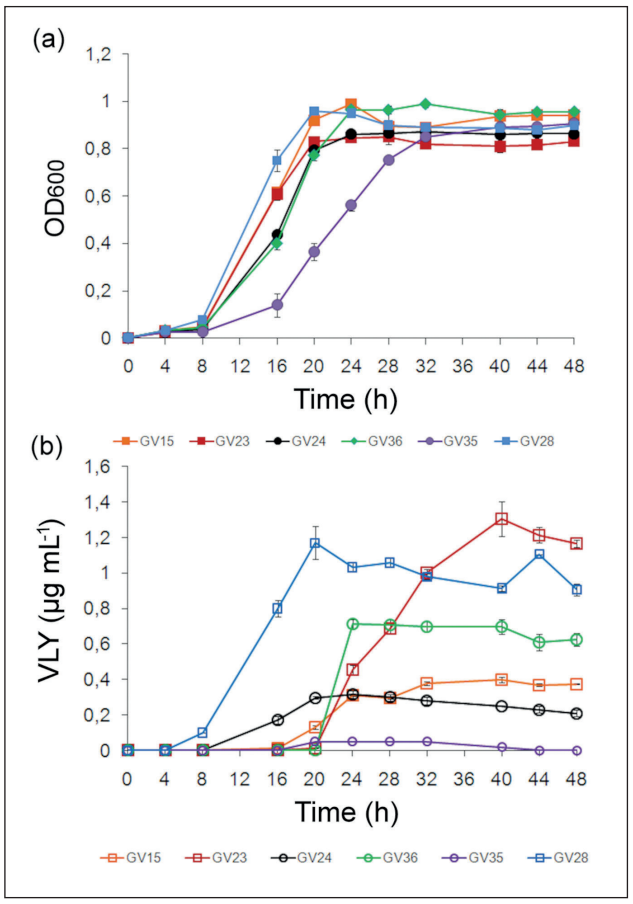


Figure 2. Detection of VLY production level in vivo. (a) Growth of six Gardnerella vaginalis isolates over time. (b) Quantitation by ELISA of VLY produced by six G. vaginalis clinical isolates at regular time intervals during growth. Error bars represent 95% CI of mean, n = 3.

Reference: Pleckaityte et al., *FEMS Immunol. Med. Microbiol.* 2012, 65(1):69-77.

Regulation of hypoxia-inducible factor HIF-3α expression via the alternative pre-mRNA splicing mechanism.

Recent genome-wide analyses of alternative splicing indicate that up to 70% of human genes may have alternative splice forms, suggesting that alternative splicing together with various posttranslational modifications plays a major role in the production of proteome complexity.

Changes in splice-site selection have been observed in various types of cancer and may affect genes implicated in tumor progression and in susceptibility to cancer. This may lead to altered efficiency of splice-site recognition, resulting in overexpression or down-regulation of certain splice variants, a switch in splice-site usage, or failure to recognize splice sites correctly, resulting in cancer-specific splice forms. At least in some cases, changes in splicing have been shown to play a functionally significant role in tumorigenesis, either by inactivating tumor suppressors or by gain of function of proteins promoting tumor development. Thus, the identification of cancer specific splice forms provides a novel source for the discovery of diagnostic or prognostic biomarkers and tumor antigens suitable as targets for therapeutic intervention.

Hypoxia has long been recognized as a common feature of solid tumours and a negative prognostic factor for response to treatment and survival of cancer patients. Biological responses to hypoxia involve induction of transcription of a network of target genes, a process which is coordinately regulated by hypoxia-inducible transcription factors (HIFs). three structurally related bHLH transcription factors (HIF-1, HIF-2 and HIF-3). HIFs recognize hypoxia response elements of targets genes as hetrodimeric complexes (HIF-1α, HIF-2α and HIF-3α) with the transcription factor Arnt.

A splice variant of HIF-3, inhibitory PAS domain protein (IPAS), inhibits the dimerization of HIF-1 and ARNT. IPAS

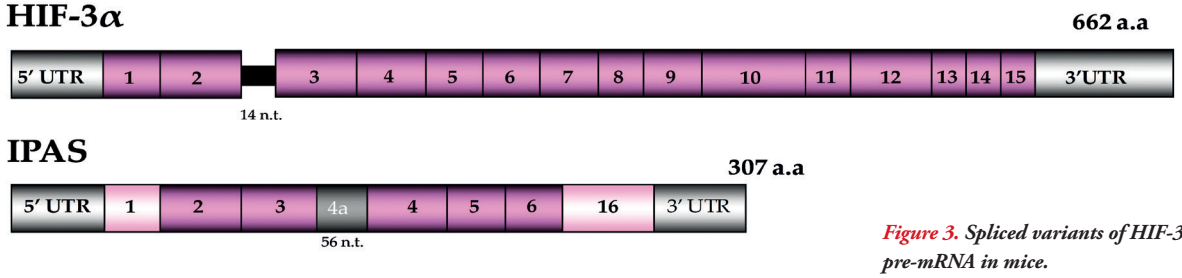


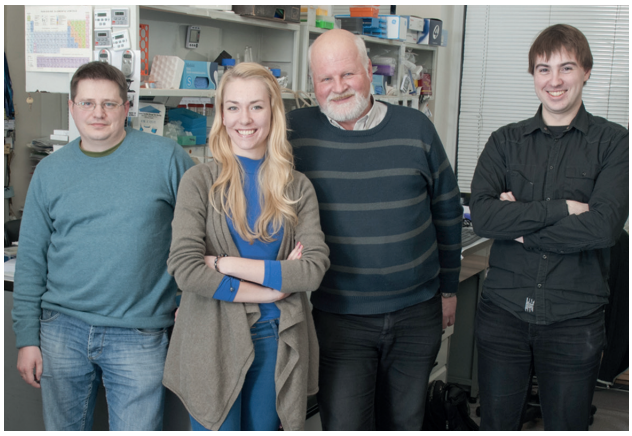
Figure 3. Spliced variants of HIF-3α pre-mRNA in mice.

protein contains a bHLH domain and a PAS domain, which are the common structures present in the HIF family. IPAS expression in hepatoma cells selectively impairs the induction of hypoxia-inducible genes regulated by HIF-1 and results in retarded tumour growth and tumour vascular density *in vivo*. In mice, IPAS was selectively expressed in Purkinje cells of the cerebellum and in the corneal epithelium of the eye. Moreover, the expression of IPAS in the cornea correlates with low VEGF gene expression under hypoxic conditions.

We established that an essential splicing factor is involved in oxygen-dependant pre-mRNA splicing regulation. Reducing cellular expression of this factor changes splicing profile to hypoxic characteristic. Overexpression of this factor moves splicing pattern to normoxic characteristic.

Thus we identified hypoxia dependable pre-mRNA splicing regulator which might reprogram cellular events and could not only be useful for the potential therapeutic applications but also for their application as an analytic tool.

This work was supported by the Framework 7 program (project *Metoxia*).



From left to right MoBiLi project scientist Dr. S.Laurinavičius, master student A.Ščerbakovaitė, group leader Dr. A.Kanopka and bioengineer L.Vilys

Molecular epidemiology of *Mycobacterium tuberculosis*

Tuberculosis (TB) caused by *M. tuberculosis* complex bacteria remains a serious health problem in Lithuania. The rates of incidence, particularly multidrug-resistant tuberculosis (MDR TB) are one of the highest in the European Society. The median survival for MDR TB patients during 2002-2008 year in Lithuania was 4.1 year (Balabanova et al, BMJ Open, 2011). The aim of this ongoing project is to characterize in detail population of *M. tuberculosis* that circulates in Lithuania including the genetic determinants of drug resistance. The research was carried out in close collaboration with Infectious Diseases and Tuberculosis Hospital, affiliate of public institution Vilnius University Hospital Santariskiu Klinikos. Genotyping of *M. tuberculosis* clinical strains was performed by reference techniques (24-locus MIRU-VNTR typing, spoligotyping) and polymorphisms of *M. tuberculosis* genome were identified by direct sequencing.

In 2011-2012, we continued genotyping of *M. tuberculosis* strains recovered from TB patients living in Vilnius. Overall, the results of this study confirmed that stabilization of TB situation is occurring. However, many of most dangerous transmission chains have not been yet broken. Moreover, the new clusters consisting of MDR and extensively drug resistant (XDR) strains are emerging. In the frame of drugs resistance, we focussed in search for the mutations associated with resistance to aminoglycosides, capreomycin, and pyrazinamide including the search for the novel targets involved in resistance outside of well-known hot spot regions of the *M. tuberculosis* chromosome. The results demonstrated that the mutations causing kanamycin resistance, particularly in the 3' region of the *rrs* gene and in the promoter of the *eis* gene could be an important driving force for the emergence and spread of XDR TB strains in Lithuania. Identification of the mutations in the *pncA* gene could be useful tool for the detection of resistance to pyrazinamide and could serve as auxiliary subtyping technique for a better differentiation of drug-resistant strains as well.

This work was supported by European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement FP7-223681 and the Agency for Science, Innovation and Technology (MITA) under grant agreements 31V-87 and 31V-99.

Reference: de Beer et al., *J. Clin. Microbiol.* 2012, 50(3):662-9.

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Funding

EC Framework 7th Programme
European Social Fund Under THE Global grant Measure
Research Council of Lithuania
Agency for Science, Innovation and Technology

Research Contracts

UAB Fermentas/Thermo Fisher Scientific, Lithuania
Abcam Ltd, Cambridge, UK
Santa Cruz Biotechnology, USA

Patents

Zvirbliene A., **Gedvilaite A,** Ulrich R., **Sasnauskas K.**
Process for the production of monoclonal antibodies.
US 7919314 B2. 2011/04/05

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The Department of Biothermodynamics and Drug Design (DBDD) was established in 2006 in the place of the former Laboratory of Recombinant Proteins. The DBDD designs novel chemical compounds as anticancer agents. The efficiency of both naturally occurring and synthetic compounds is evaluated by structural biothermodynamics and molecular modelling methods. The laboratory's personnel consists of five teams according to their research activities:

The Team of Molecular and Cell Biology, headed by Dr. Jurgita Matulienė (Ph. D. in cell biology from the University of Minnesota, USA, 2003), produces drug target proteins by gene cloning, expression in *E.coli*, insect, or mammalian cells, and chromatografic purification of large quantities of active proteins sufficient for biothermodynamic measurements of compound binding. Several projects involve the design of protein domain constructs. Live human cancer cells are cultured for the evaluation of compound anticancer activity. Dr. Vilma Petrikaitė has a Ph. D. in pharmacy and performs compound testing in mice xenografts.

The Team of Organic Synthesis, headed by Dr. Virginija Dudutienė (Ph. D. in organic synthesis from the Vilnius University, 2005), synthesizes compounds that are designed to bind carbonic anhydrases and other drug target proteins. Compounds are designed by computer docking, molecular modelling, and comparison with naturally occurring or previously synthesized compounds. The special interests and capabilities of the group are in the field of synthesis of compounds with multiple conjugated aromatic heterocycles.

The Team of Biophysics, headed by Dr. Daumantas Matulis (Ph. D. in biochemistry, molecular biology and biophysics from the University of Minnesota, USA, 1998), measures compound binding to target proteins by isothermal titration calorimetry (ITC), thermal shift assay (ThermoFluor®), and pressure shift assay (PSA). The team performs the characterization of protein stability in the presence of various excipients and the measurements of target protein enzymatic activity.

The Team of Computer Modelling, headed by Vytautas Petrauskas (Ph. D. in physics from the Vilnius University, 2008), is responsible for the *in silico* docking of large compound libraries and the analysis of X-ray crystal structures of synthetic compound – protein complexes solved in collaboration with Dr. Saulius Gražulis' group in the Department of Protein – DNA interactions. Molecular modelling of candidate compounds often predicts novel compounds with improved binding capabilities. The group, together with several collaborating scientists is developing the software that estimates the energetics of compound binding to a protein when only the crystal structure of the free protein is available.

The Team of Amyloid Research

Recently a new team has started upon the return of Dr. Vytautas Smirnovas (Ph. D. from the Technical University of Dortmund, 2007) to Lithuania in 2011. The main interests and research of his team lie in the protein aggregation and amyloidogenesis that are involved in a number of diseases, including such neurodegenerative disorders as Alzheimer's and Parkinson's. His research is described in greater detail in the MoBiLi section of this issue.

MoBiLi project scientist Vytautas Smirnovas with students Akvilė Botyriūtė and Katažyna Milto

Research projects

Several protein targets have been selected for the investigation of protein – compound binding thermodynamics and the design of novel compounds with desired properties. A family of human carbonic anhydrases [1, 8], heat shock protein Hsp90 [2, 4, 5, 13, 14], and several epigenetically important proteins [3] were chosen as anticancer drug targets.

Novel methods and thermodynamic approaches are being used and developed in the laboratory. Detailed thermodynamic description of natural compound – protein interaction provides clues to improved compound affinity and specificity. In addition to the Gibbs free energy, enthalpy, entropy, and the heat capacity, the laboratory studies the volume and compressibility of the protein – compound interactions [9, 10].

The laboratory is interested in the fundamental thermodynamics of the hydrophobic effect [7] and the development of thermodynamic methodology for compound – protein interactions [11, 12].

Carbonic anhydrases as anticancer drug targets

Carbonic anhydrases (CAs), a group of zinc containing enzymes, are involved in numerous physiological and pathological processes, including gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity and the growth and virulence of various pathogens. In addition to the established role of CA inhibitors as diuretics and antiglaucoma drugs, it has recently emerged that CA inhibitors could have potential as novel anti-obesity, anticancer, and anti-infective drugs (Supuran, 2008, 2012). CAs catalyze the conversion of CO_2 to the bicarbonate ion and protons. There are 12 catalytically active CA isoenzymes in humans. A number of CA inhibitors, mostly unsubstituted sulfonamides, have already been designed. However, most present inhibitors are insufficiently selective for targeting CA isozymes, such as hCAIX and hCAX-II, which are anticancer targets.

Here at the DBDD we have cloned and purified most cytoplasmic CAs and catalytic domains of transmembrane CAs. The organic synthesis team, together with collaborators, designed and synthesized over 500 novel compounds that bind CAs with submicromolar to subnanomolar affinity. Several novel series of CA inhibitors exhibited extremely tight affinity and an appreciable selectivity towards selected CA isozymes [1, 8].

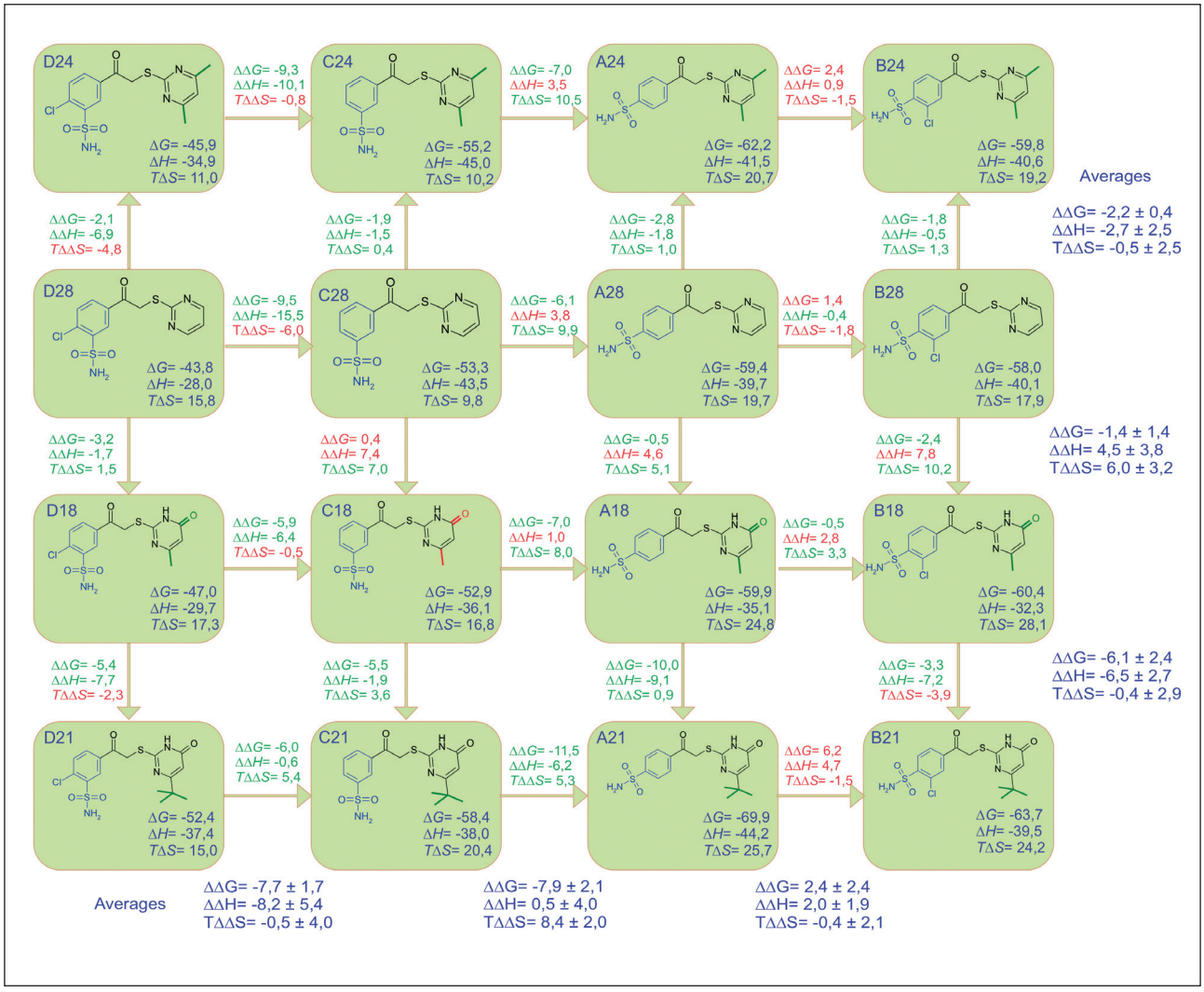


***Figure 1.** Additivity of the intrinsic thermodynamic parameters of compound binding to CA I (kJ/mol at 37 °C). Numbers at the compounds show the binding parameters while the numbers at the arrows show the differences. Averages of the differences are listed on the right and below the figure.*

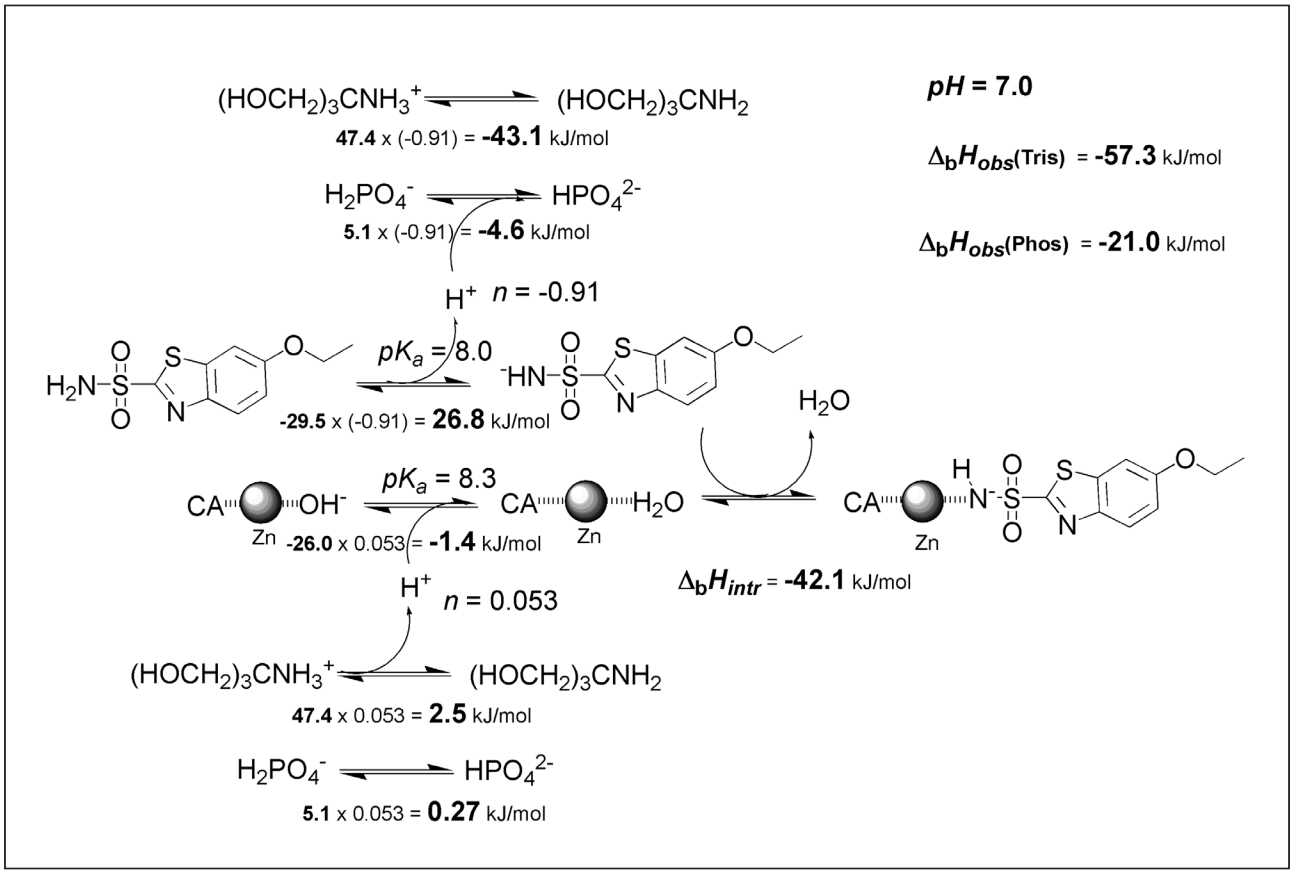


Figure 2. Contributions from linked reactions to the intrinsic binding enthalpy of ethoxzolamide to recombinant human CA XIII [8].

Ligand binding to proteins at high pressure

The volume changes accompanying ligand binding to proteins are thermodynamically important and potentially could be used in the design of compounds with specific binding properties. Measuring the volumetric properties could yield as much information as the enthalpic properties of binding. Pressure-based methods are significantly more laborious than temperature methods and are underused. The pressure shift assay (PressureFluor, analogous to the ThermoFluor, thermal shift assay) uses high pressure to denature proteins. The PressureFluor method was used to study the ligand binding thermodynamics of Hsp90 and human serum albumin. Ligands stabilize the protein against pressure denaturation, similar to the stabilization against temperature denaturation.

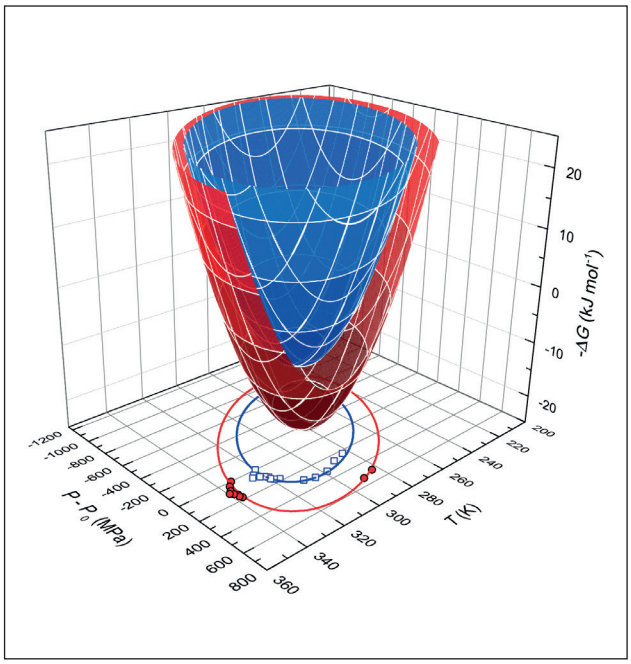


Figure 3. The Gibbs free energy dependence on pressure and temperature. Inner surface represents the ligand free Hsp90N stability region, while outer surface shows stability region of protein-ligand system with 200 μM of added ligand (Petrauskas et al 2013).

Inhibition of the Hsp90 chaperone

Heat shock protein 90 (Hsp90) is a molecular chaperone that is responsible for the correct folding of a large number of client proteins. The client proteins include many overexpressed oncogenes that are critical for the transformed phenotype observed in tumours.

Our laboratory is interested in the thermodynamics of inhibitor binding. A series of Hsp90 inhibitors were designed that exhibit extremely tight subnanomolar affinities. Intrinsic thermodynamics of their binding and the cocrystal structures were determined [2, 4]. Volumetric properties at high pressures were determined for the compound [10, and Petrauskas et al. 2013]. The EU and US patents have been obtained for the series.

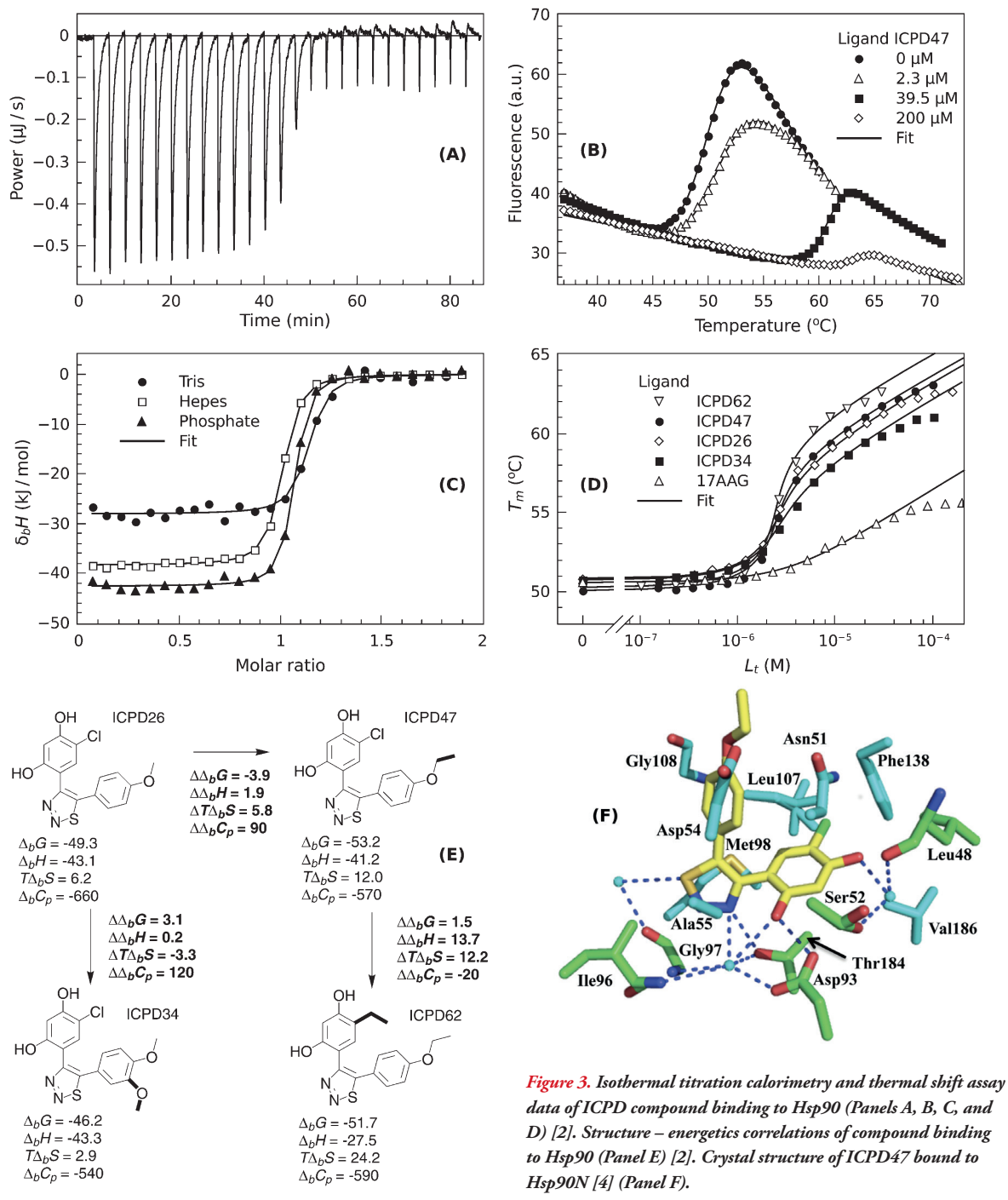
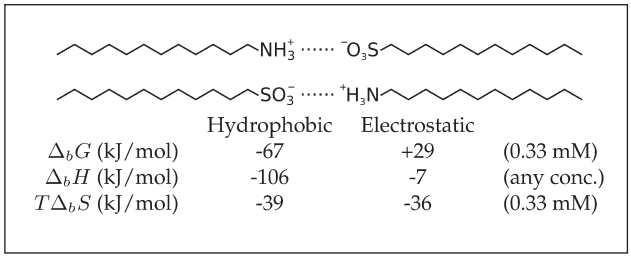


Figure 3. Isothermal titration calorimetry and thermal shift assay data of ICPD compound binding to Hsp90 (Panels A, B, C, and D) [2]. Structure – energetics correlations of compound binding to Hsp90 (Panel E) [2]. Crystal structure of ICPD47 bound to Hsp90N [4] (Panel F).

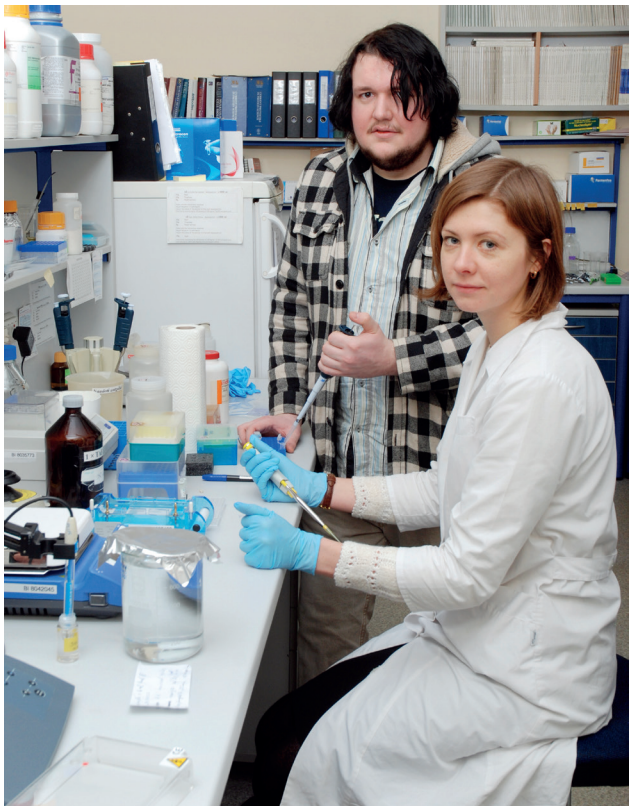
Thermodynamics of the Hydrophobic effect

The energetics of the hydrophobic effect is of fundamental importance to biophysics. It is important for the understanding of protein folding, ligand binding, and the formation of lipid membranes. The common view emphasizes entropic origins of the binding force of the hydrophobic effect. In our previous studies (Matulis and Bloomfield, 2001), we have shown the importance of enthalpy and phase changes in the system of long chain aliphatic compounds. The binding of oppositely charged detergents shows similar signatures of the hydrophobic forces.



	Hydrophobic	Electrostatic	
$\Delta_b G$ (kJ/mol)	-67	+29	(0.33 mM)
$\Delta_b H$ (kJ/mol)	-106	-7	(any conc.)
$T\Delta_b S$ (kJ/mol)	-39	-36	(0.33 mM)

Figure 4. Energetics of dodecylammonium binding to dodecane sulfonate forming solid aggregate [7].



Junior scientist Vaida Jogaitė and PhD student David Daniel Timm performing molecular biology experiments

Master student Sandra Bakšytė performing PCR experiment

Services

The DBDD is seeking to license out the compounds described in patents and patent applications. The DBDD is interested in collaborations where our expertise in recombinant protein production and the determination of compound – protein binding thermodynamics and recombinant protein stability characterization could be applied. Protein – ligand binding constants and protein thermal stability profiles at hundreds of conditions may be determined in a single experiment by consuming microgram quantities of protein.

Conferences

The DBDD regularly participates in many international conferences and symposiums, including:
International Conference on the Hsp90 chaperone machine
International Conference on the Carbonic Anhydrases
International Conference on High Pressure Bioscience and Biotechnology
Biothermodynamics Symposium
European Biophysics Congress
Biophysical Society Annual Meeting
Gibbs Conference on Biothermodynamics
International Conference of Lithuanian Biochemical Society
COST project CM0804 and TD0905 meetings

Collaboration

The DBDD has ongoing collaborations with a number of research laboratories and industry worldwide, including:
Institute of Medical Technology, University of Tampere, Finland
University of Florence, Italy
International Institute of Molecular and Cell Biology, Warsaw, Poland
Jensen Pharmaceuticals, Johnson&Johnson, USA
Centre for Structural Biochemistry, Montpellier, France
Institute of Organic Synthesis, Riga, Latvia
Institute of Organic Chemistry, University of Tubingen, Germany
Cancer Research Centre, University of Edinburgh, UK
St. Andrews University, UK
Institute of Chemistry, UMR CNRS 7272, Nice, France
Faculty of Chemistry, Vilnius University, Lithuania
Faculty of Natural Sciences, Vilnius University, Lithuania
Institute of Biochemistry, Vilnius, Lithuania
Lithuanian University of Agriculture, Kaunas, Lithuania
AB "Amilina", Panevėžys, Lithuania
Nature Research Centre, Institute of Botany, Vilnius, Lithuania

Funding

EC Framework 7th Programme
European Social Fund under the Global Grant Measure
Research Council of Lithuania

Patents and Patent Applications

Matulis D., Dudutienė V., Matulienė J. and **Mištinaitė L.** Benzimidazo[1,2-C][1,2,3]Thiadiazol-7-Sulfonamides as Inhibitors of Carbonic Anhydrase and the Intermediates for Production Thereof. EP2054420

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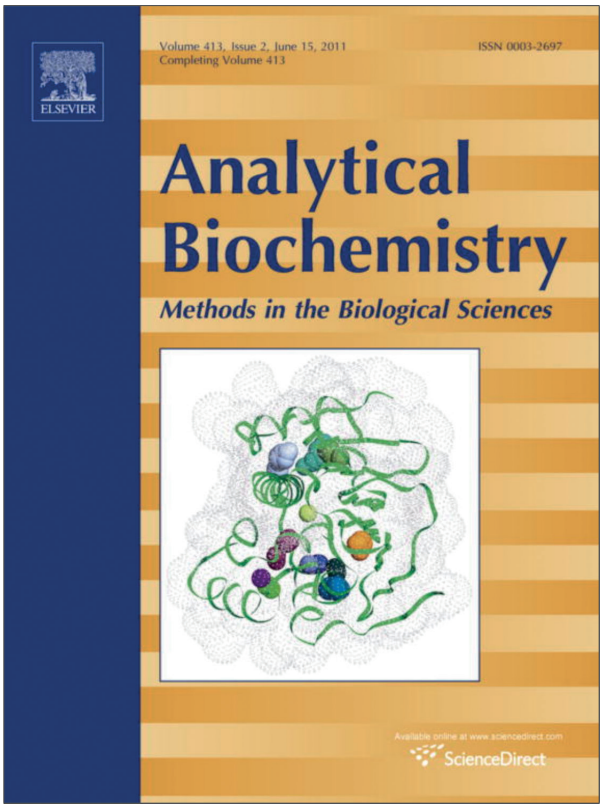

The cover of the Analytical Biochemistry issue showing our picture from publication 10

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At present computational methods are playing an increasingly important role in biological research. Breakthroughs in technologies have resulted in a flood of various types of biological data such as genome sequences for different organisms, data on gene expression, protein-protein interactions, etc. Computational biology and bioinformatics are helping to make sense of all this vast biological data by providing tools for performing large-scale studies. In addition, computational biologists are utilizing available experimental data to improve various analytical and predictive methods that could help address specific biological problems.

Research carried out in our department covers a broad range of topics that together can be described as *Computational Studies of Protein Structure, Function and Evolution*. There are two main research directions:

- Development of methods for the detection of protein homology from sequence data, comparative modeling, analysis and evaluation of protein three-dimensional structure.
- Application of computational methods for discovering general patterns in biological data, structural/functional characterization of proteins and their complexes; design of novel proteins and mutants with desired properties. We address a variety of challenging biological problems, yet our main focus is on proteins and protein complexes involved in DNA replication, repair and recombination.

Development of computational methods

During the report period our major efforts in methods development were devoted to protein homology detection and evaluation of protein structure.

The concept of homology (common evolutionary origin) is at the heart of most studies dealing with protein sequence, structure and function. In the absence of three dimensional (3D) protein structures the homology detection has to rely on sequence data. Currently, the most sensitive homology inference methods are based on comparison of multiple sequence alignments represented as sequence profiles. However, these profiles can be constructed, compared and scored in many different ways. On the one hand this complicates the development of profile-based homology detection methods; on the other hand this means a lot of space for improvement. During the two years covered by this report we have been actively exploring different paths to improve the profile-based homology detection. At present, we are in the final stages of the development of a new competitive homology detection method.

The evaluation of protein structure is particularly important in computational protein modeling. Scoring models against the native structure is at the heart of development and benchmarking of protein structure prediction and refinement methods. It may seem that one-to-one correspondence between computational models and the native (reference) structure should make such evaluation trivial. Yet, contrary to this view, it is an open problem, because many aspects of the reference-based model evaluation still lack desired robustness. We have been actively researching how to improve the reference-based model evaluation. Our attempts resulted in a new highly effective score, which is described in more detail below.

CAD-score: evaluation of protein structural models based on contact area difference

CAD-score (**C**ontact **A**rea **D**ifference score) is a new evaluation function quantifying differences between physical contacts in a model and the reference structure. It uses the concept of residue-residue contact area difference (CAD) introduced by Abagyan & Totrov (J. Mol. Biol. 1997; 268:678–685). Contact areas, the underlying basis of the score, are derived using the Voronoi diagram of spheres that correspond to heavy atoms of van der Waals radii (Figure 1). The Voronoi diagram of spheres is constructed by a new algorithm that is especially suited for processing macromolecular structures.

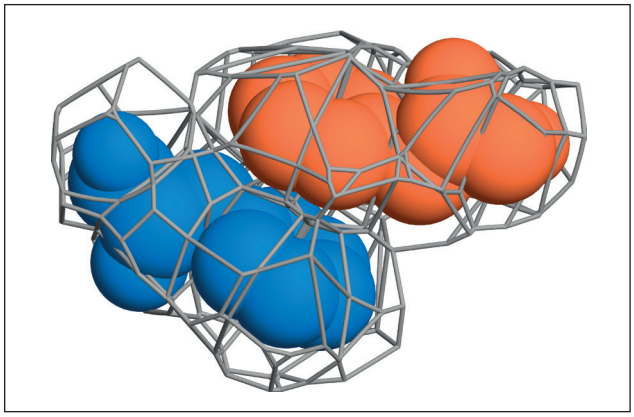

***Figure 1.** Voronoi diagram of spheres of a residue pair*

The algorithm resolves residue–residue contacts at the level of atoms, making it possible to consider contacts not only between entire residues but also between subsets of residue atoms (main chain, side chain) (Figure 2).

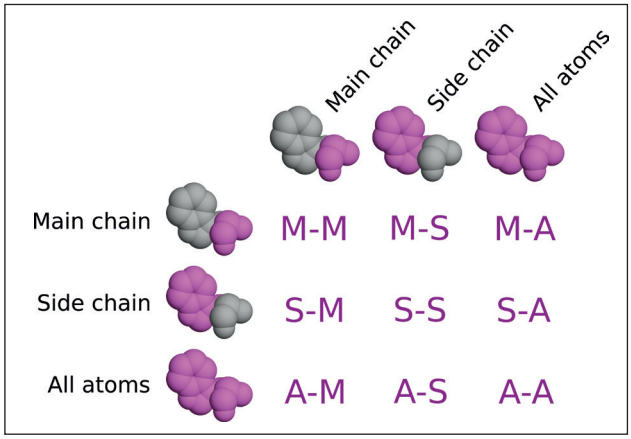

Figure 2. Contact types between residues

CAD-score has a number of attractive properties. It is based on physical contacts between residues, thereby directly reflecting interactions within the protein structure. It is a continuous, threshold-free function that returns quantitative accuracy scores within the strictly defined boundaries. The definition of CAD-score does not contain any arbitrary parameters. On single-domain structures CAD-score is highly correlated with GDT-TS, a commonly accepted model evaluation score (Fig. 3). This should be expected for any effective evaluation score. However, the advantage of CAD-score over GDT-TS is in that it displays a stronger emphasis on the physical realism of models and provides a better resolution of models that have similar accuracy of the main chain (Fig. 4).

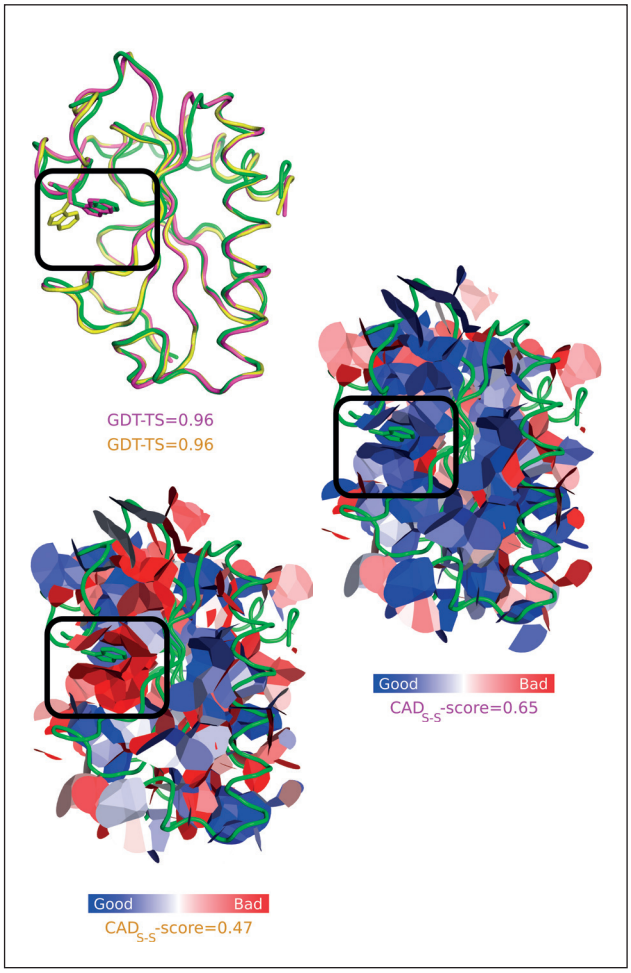

***Figure 4.** An example of improved model quality resolution by CAD-score compared to that by GDT-TS. Two models (pink and gold) have identical GDT-TS values (left), but very different CAD-score values because of the different accuracy of side chain prediction.*

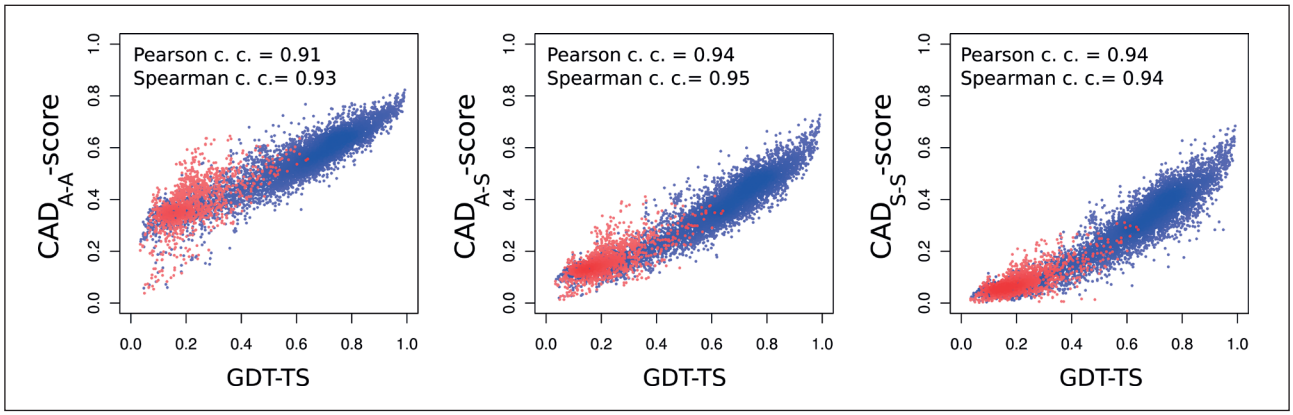

***Figure 3.** Correlation between GDT-TS and three types of CAD-score on a large number of models. In all three cases correlation coefficients exceed 0.9.*

In contrast to GDT-TS, our new score provides a balanced assessment of domain rearrangement, removing the necessity for different treatment of single-domain, multi-domain and multi-subunit structures. Moreover, CAD-score makes it possible to assess the accuracy of inter-domain or inter-subunit interfaces directly. In addition, the approach offers an alternative to the superposition-based model clustering.

We believe that all these attractive properties will make CAD-score a valuable tool for the community of computational biologists at large. In fact, CAD-score has already attracted atten-

tion of experts in the field. The organizers of CASP10 (large-scale protein structure prediction experiment) conducted during the summer of 2012 have included CAD-score into the "portfolio" of model evaluation scores (www.predictioncenter.org/casp10).

The CAD-score has been published in "Proteins" [7] and its implementation is available both as a web server and a standalone software package. CAD-score and all the other methods developed in our laboratory can be accessed through our website at: http://www.ibt.lt/bioinformatics/software/.

Application of computational biology methods to specific biological problems

An important element of our research are projects in which computational methods alone or combined with experiments (in collaboration with experimental labs) are applied to address specific biological questions. The application of computational methods is not limited to some specific research area. Nevertheless, most of these research projects involve proteins participating in DNA metabolism, in particular in DNA replication and repair. Some of the projects executed during the reported period or those still ongoing are listed below.

- Computational analysis of the nature and distribution of DNA replication proteins in genomes of double stranded DNA viruses
- Computational analysis of evolutionary distribution and structural properties of bacterial DNA polymerase III catalytic subunits and their homologs
- Computational analysis of type I restriction-modification systems (collaboration with Prof. Janusz Bujnicki, International Institute of Molecular and Cell Biology, Warsaw)
- Analysis of rare missense mutations of human chorionic gonadotrophin, a hormone, determining the success of early pregnancy (collaboration with Prof. Maris Laan, University of Tartu)
- Molecular mechanisms of M. tuberculosis DNA mutagenesis and the role of vitamin B12 in mycobacterial pathogenesis (collaboration with Prof. Valerie Mizrahi and Dr. Digby Warner at University of Cape Town)
- Computational/experimental studies of molecular functions of Elg1, a protein involved in the maintenance of genome stability in eukaryotes (collaboration with Prof. Martin Kupiec, Tel Aviv University)

Two of these research projects are described in more detail below.

Computational analysis of DNA replication proteins in double-stranded (ds) DNA viruses

Free-living cellular organisms for their genome replication always encode a DNA replicase consisting of a DNA polymerase, a DNA sliding clamp and a clamp loader. In contrast, some dsDNA viruses do not even have their own DNA polymerases. Could it be that the size of a genome is an important factor determining the need for a processive DNA replicase? Perhaps there is an approximate genome size threshold, above which the processivity properties of a replicase become critical? We considered that dsDNA viruses may hold a key to the answers of these fundamental questions and performed the analysis of all available genomes of dsDNA viruses. Indeed, we discovered previously unnoticed relationship between DNA replicases and the genome size. As the genome size increases, viruses universally encode their own DNA polymerases. Homologs of DNA sliding clamps and clamp loader subunits produce another highly non-random presence/absence pattern. The absence of sliding clamps in large viral genomes usually coincides with the presence of atypical polymerases. Meanwhile, sliding clamp homologs, not accompanied by clamp loaders, have an elevated positive electrostatic potential, characteristic of non-ring viral processivity factors that bind the DNA directly. This observation has implications not only for viral DNA sliding clamps but also for human Hus1 and Rad9, subunits of the ring shaped 9-1-1 DNA damage checkpoint complex. (Fig. 5). Most importantly, the results of our study have predictive power regarding DNA replicases in newly determined genomes of dsDNA viruses. In addition, it appears that our findings for dsDNA viral genomes may be extended to symbiotic bacteria having extremely reduced genomes. The study has been published in "*Nucleic Acids Research*" [1].

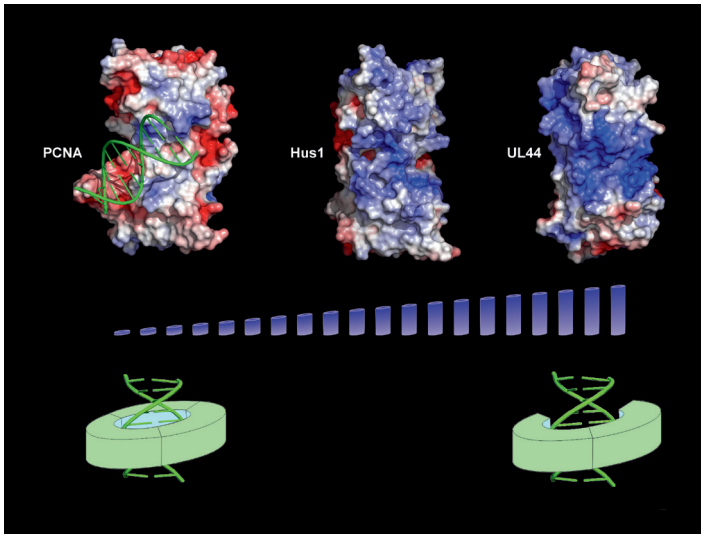

Figure 5. ***Correlation between high electrostatic potential and the non-ring architecture of DNA sliding clamps. Yeast PCNA has a closed ring, human cytomegalovirus UL44 is an open dimer. Human Hus1 is part of the ring-shaped 9-1-1 complex, but perhaps may also bind DNA as a monomer.***

Analysis of rare missense mutations of human chorionic gonadotrophin (hCG) β-subunit

In collaboration with Prof. Maris Laan (University of Tartu, Estonia) we explored the impact of several rare missense mutations of human chorionic gonadotrophin (hCG) *β*-subunit. Placental hCG is one of the key hormones determining success of early pregnancy. Like other heterodimeric glycoprotein hormones (luteinizing hormone, follicle-stimulating hormone and thyroid-stimulating hormone), hCG is formed by non-covalent association of the common *α*-subunit and the hormone-specific *β*-subunit. During previous survey of over a thousand of North-Europeans, three rare mutations (Val56Leu, Arg8Trp and Pro73Arg) within hCG *β*-subunit were identified. Using combination of computational (sequence and structure analysis, molecular dynamics) and experimental (co-immunoprecipitation, immuno- and bioassays) approaches we assessed the impact of these mutations on the structure and function of hCG. In computational assessment the Val56Leu mutation displayed the most dramatic impact on the assembly of the intact hCG. This finding was confirmed by experiments showing that compared to the wild-type only about 10% of the mutant hCG*β* assembled into secreted intact hCG. However, interestingly, the effect of poor dimerization was compensated by a much stronger signaling response triggered upon binding to the hormone receptor. Another mutant (Pro73Arg) showed an increased propensity towards alternative conformation, but no visible effect on its biological activity. Computational analysis of the third mutation (Arg8Trp) did not reveal any significant alterations in the assembly of intact hCG as also confirmed by experiments. In summary, the study suggested that only mutations with neutral or mild functional consequences might be tolerated in the major hCGβ coding genes, CGB5 and CGB8. Results of the study [6] were reported as a cover story in "*Molecular Human Reproduction*" (Fig. 6).

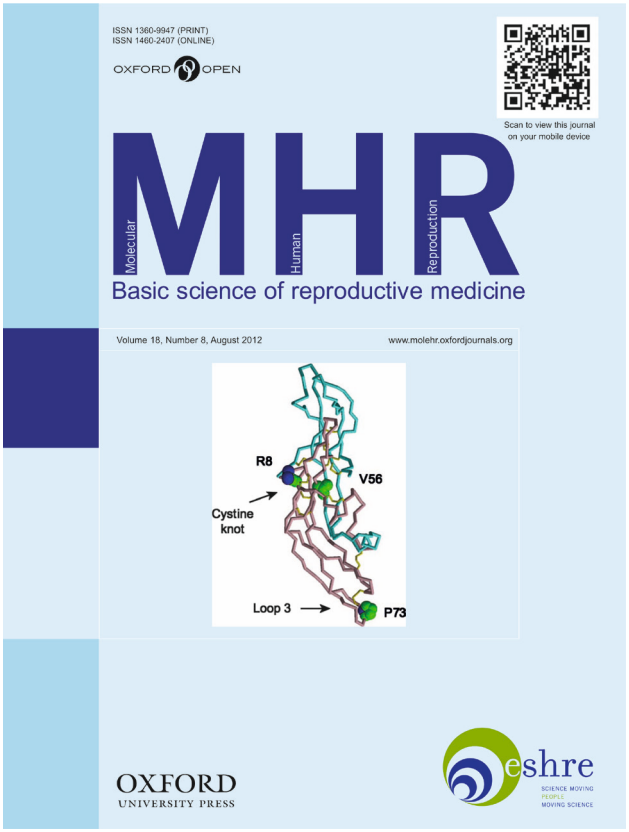

Figure 6. ***The MHR cover displaying the hCG structure with three residues affected by the identified mutations shown in the space-filling representation.***

Collaborative interactions

In addition to scientific interactions with our colleagues within the Institute of Biotechnology we are involved in a number of external collaborations:

Prof. Penny Beuning, Northeastern University, Boston, MA, USA
Prof. Maris Laan, Institute of Molecular and Cell Biology, University of Tartu, Estonia
Prof. Valerie Mizrahi & Dr. Digby Warner, University of Cape Town, South Africa
Prof. Arvydas Skeberdis, Institute of Cardiology, Lithuanian University of Health Sciences, Kaunas, Lithuania
Prof. Janusz Bujnicki, International Institute of Molecular and Cell Biology, Warsaw, Poland
Prof. Martin Kupiec, Tel Aviv University, Israel

Funding

EC Framework 7th Programme
Howard Hughes Medical Institute
Research Council of Lithuania

Selected Publications 2011-2012

1. **Kazlauskas D.**, **Venclovas Č**: Computational analysis of DNA replicases in double-stranded DNA viruses: relationship with the genome size. *Nucleic Acids Res.* 2011, 39(19):8291-305.
2. **Laganeckas M.**, **Margelevičius M.**, **Venclovas Č**: Identification of new homologs of PD-(D/E)XK nucleases by support vector machines trained on data derived from profile-profile alignments. *Nucleic Acids Res*. 2011, 39(4):1187-96.
3. **Olechnovič K.**, **Margelevičius M.**, **Venclovas Č**: Voroprot: an interactive tool for the analysis and visualization of complex geometric features of protein structure. *Bioinformatics* 2011, 27(5):723-4.
4. **Timinskas K.**, **Venclovas Č**: The N-terminal region of the bacterial DNA polymerase PolC features a pair of domains, both distantly related to domain V of the DNA polymerase III τ subunit. *FEBS J*. 2011, 278(17):3109-18.
5. **Kazlauskas D.**, **Venclovas Č**: Two distinct SSB protein families in nucleo-cytoplasmic large DNA viruses. *Bioinformatics* 2012, 28(24):3186-90.
6. Nagirnaja L., **Venclovas Č.**, Rull K., Jonas K.C., Peltoketo H., Christiansen O.B., **Kairys V.**, Kivi G., Steffensen R., Huhtaniemi I.T., Laan M: Structural and functional analysis of rare missense mutations in human chorionic gonadotrophin beta-subunit. *Molecular human reproduction* 2012, 18(8):379-90.
7. **Olechnovič K.**, **Kulberkytė E.**, **Venclovas Č**: CAD-score: A new contact area difference-based function for evaluation of protein structural models. *Proteins* 2013, 81(1):149-62.
8. Gopinath K., **Venclovas Č.**, Ioerger T.R., Sacchettini J.C., McKinney J.D., Mizrahi V., Warner D.F. A vitamin B_{12} transporter in *Mycobacterium tuberculosis*. *Open Biology* 2013, 3(2):120175.

Book chapters

Barsky, D., Lawrence, T.A. and **Venclovas Č.** How proteins slide on DNA // Biophysics of DNA-Protein Interactions. Editors: Williams M. C. and Maher L. J. III. Springer Science+Business Media, LLC 2011, 39-68.

Venclovas Č. Methods for sequence-structure alignment. // Methods in molecular biology. Editors: Orry Andrew J. W., Abagyan Ruben. Humana Press 2012, 857:55-82.

Sector of

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Ieva Kazlauskaitė, M.Sc.
Jurga Aleksejūnaitė, M.Sc.
Milda Aštrauskaitė, M. Sc.

Affiliated scientists
Prof. Gervydas Dienys, PhD, Dr. Habil.

Sector of Applied Biocatalysis was established in 2007 as a group of Industrial Biotechnology in conjunction with the start of the National Programme on the Development of Industrial Biotechnology in Lithuania 2007-2010. In 2010 the group was transformed into the Sector of Applied Biocatalysis and is headed by Inga Matijošytė (Ph.D. in biochemistry and biocatalysis from Delft University, The Netherlands, 2008).

Sector's research is directed towards the search for enzymes with new functionalities and their development towards applied biocatalysis. The limited number of suitable and well characterized biocatalysts delays the progress in the application of enzymes in the synthesis of compounds for materials, pharmaceuticals and chemicals.

Recently, the sector is orienting the research towards dicrovery of new novel biocatalytic routes for high-added value products from bio-based raw materials – biopolymers. Therefore, the spin-off company of the sector was established.

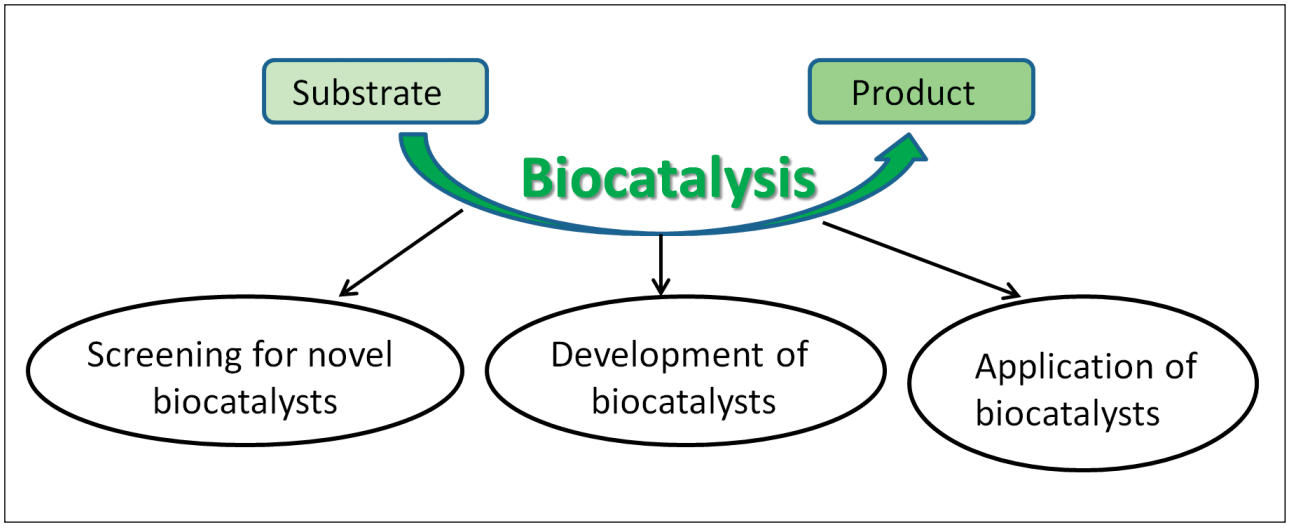

Figure 1. ***Research areas of Sector of Applied Biocatalysis***
Sector of Applied Biocatalysis seeks to identify biocatalysts with novel activities by screening for enzymes, development of biocatalyst and application of biocatalyst (Figure 1). The research focuses on developing of biocatalytic systems employing lypolytic, hydrolyti, proteolytic and oxidative enzymes. We strive to meet scientific challenges in combination with application-oriented research. Therefore, close collaboration with industrial partners resulted in several research oriented projects *(Grants).*

Collaboration

dr. Boris Kovenbach**,** University of Applied Sciences, Muttenz, Switzerland
Prof. Dr. P. Corvini, University of Applied Sciences, Muttenz, Switzerland
Prof. R.A. Sheldon, JSC CLEA technologies, the Netherlands
Prof. I.W.C.E. Arends, Delft University of Technology, the Netherlands
Nathalie Berezina, Materia Nova, R& D Centre, Belgium
dr. Patrizia Cinelli, University of Pisa, Italy

Funding

Agency for Science, Innovation and Technology

Publications

1. **I.Matijošytė**, I.W.C.E. Arends, R.A. Sheldon, S. de Vries. Preparation and use of cross-linked aggregates (CLEAs) of laccases. *J. Mol. Catal. B: Enzym*. 2010, 62:142-148.
2. S.A. Tromp, **I. Matijošytė**, R.A. Sheldon, I.W.C.E. Arends, M.T. Kreutzer, J.A. Moulijn, S. De Vries. Mechanism of laccase/TEMPO oxidation of benzyl alcohol. *ChemCatChem*. 2010, 2:827-833.
3. **A.Veteikytė, M. Aštrauskaitė, R. Gruškienė**, R. Tekorienė, **I. Matijošytė**. Secondary alcohol oxidase activity identified in genus of *Pseudomonas* isolated from the oil polluted soil. *Biocatal. Agricult. Biotechnol.* 2013, 10.1016/j.bcab.2012.11.005

DNA Sequencing Center

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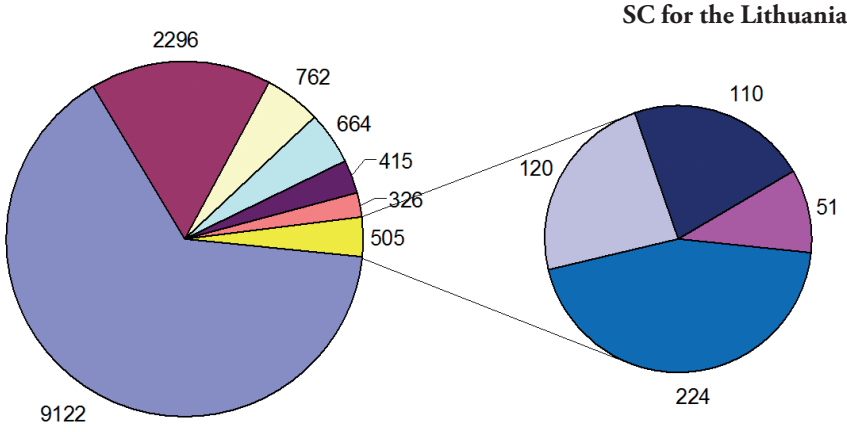
DNA Sequencing Center (SC) of the Institute of Biotechnology (IBT) is successfully running since March 27 of 2003. SC was founded to help researchers, both at IBT as well as other institutions in Lithuania, process DNA samples in an efficient and economical manner. The Center is equipped with the Applied Biosystems 3130*xl* Genetic Analyzer 16-capillary automated DNA sequencer that yields 700 to 1000 bases per template. It performs cycle sequencing reactions using fluorescent dye terminators ABI Big Dye® Terminator v3.1 on any kind of DNA (plasmid, phage or PCR product) provided by the users. We also run the user's reactions. Usually, turnaround time takes 2-3 days after the receipt of samples. Sequencing of the larger samples may take longer.

Services provided by the DNA SC include:

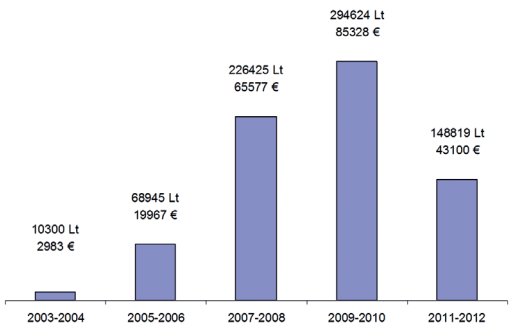
* Custom DNA Sequencing
* Sequencing, PCR troubleshooting and training workshops

We are committed to giving every user satisfactory sequence.

Number of DNA sequencing reactions performed by SC for the Lithuanian institutions in 2011- 2012



Sequencing Income for 2003- 2012



Start-ups

UAB Baltymas is a small Lithuanian start-up biotechnology company. Founded in 2011 by young scientists of the Institute of Biotechnology (Vilnius) it combines scientific experience and knowledge for generation of novel biotechnology products. The company develops yeast expression systems and employs them for synthesis of native recombinant proteins. UAB Baltymas can offer customers yeast-expressed viral nucleocapsid proteins for use in diagnostics of viral infection. It can also propose recombinant human cell proteins both for fundamental and applied research and for biopharmaceutical purposes. It is also open for synthesis of various recombinant proteins in yeast on a contract basis. For more information please visit company website at www.baltymas.lt and contact by e-mail: info@baltymas.lt

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"IMD Technologies" is a start-up company, established in 2012 under the High Technology Development programme in Lithuania for 2011-2013 "R&D Commercialization" project which was funded by Agency of Science, Innovation and Technology (MITA). The company is based on the premises of Vilnius University Institute of Biotechnology.

"IMD technologies" is R&D performing SME with the goal to fulfill industrial need to replace the conventional (chemical) methodologies with green alternatives by offering designed enzymatic and/or green technologies.

"IMD Technologies" performing R&D and produces biopolyols, which are used for production of polyurethane foam. Current polyols which are used in industry are petroleum-based products. "IMD Technologies" proposes biopolyols made from vegetable oils. These biopolyols have much better characteristics (natural, not toxic, anti-allergic, biodegradable) compared with chemical polyols including reduction of polyurethane fire potential to "green" biopolyol basis.

Also, "IMD Technologies" produces epoxidized oil, which can be used as natural plasticizer instead of chemical plasticizers in such industries as: cosmetics, PVC production, toys manufacturing, dyeing and polishing products manufacturing and etc. Epoxidized oil can change phthalates, which are toxic and dangerous for health and soon will be forbidden in EU to be used as plasticizers. Moreover, epoxidized oil can be used instead of any other plasticizers.

Furthermore, "IMD Technologies" is developing biocatalysts with novel activities by the three most common ways: screening for enzymes (environmental samples, enzyme and strain collections, metagenomic and expression databases), development of biocatalyst (gene engineering, development of analytical systems) and application of biocatalyst (immobilization, recycling, proof of principal, activity/selectivity, stability, reaction media). Company can also carry out and develop various microbiological and chemical analytical methods.

Company has a professional and experienced team, which has a long term experience in biotechnology field, including participation in various international and national R&D projects (FW7, Eureka and etc.).

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