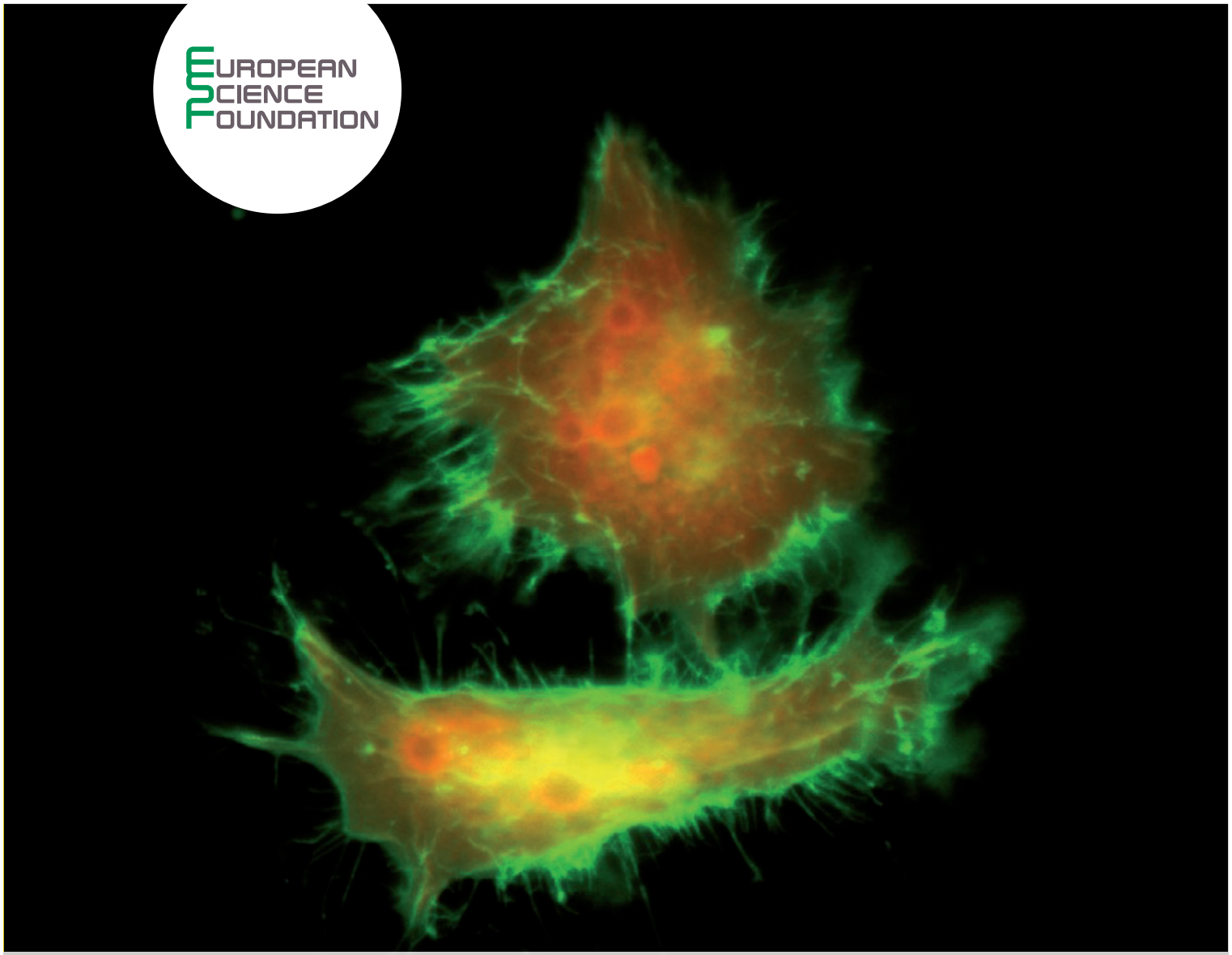

ESF EUROCORES Programme

Membrane Architecture and Dynamics (EuroMEMBRANE)

Final Report

European Science Foundation (ESF)

The European Science Foundation (ESF) was established in 1974 to provide a common platform for its Member Organisations to advance European research collaboration and explore new directions for research. It is an independent organisation, owned by 67 Member Organisations, which are research funding organisations, research performing organisations and academies from 29 countries. ESF promotes collaboration in research itself, in funding of research and in science policy activities at the European level. Currently ESF is reducing its research programmes while developing new activities to serve the science community, including peer review and evaluation services.

www.esf.org

ESF Standing Committee for Life, Earth and Environmental Sciences (LESC)

The ESF Standing Committee for Life, Earth and Environmental Sciences (LESC) is one of five disciplinary scientific committees, composed of leading scientists nominated by the Member Organisations.
LESC is responsible for identifying scientific priorities, formulating strategies and developing research agenda within its scientific domains. The Committee aims to better understand biological, environmental and Earth systems across time and space. It covers activities from molecular and systems biology to global change of the environment.
LESC covers the following scientific domains:

- Molecular Biosciences
- Microbiology
- Biological Chemistry
- Agriculture
- Plant and Animal Biology
- Ecology
- Climate Research
- Earth Sciences
- Glaciology
- Oceanography
- Meteorology

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Cover Image:
Cells were engineered to express membrane docking sites (in green) for a lipid-modifying enzyme localised in the cytosol. Translocation of lipid kinases and phosphatases can thus be targeted chemically to specific sub-membrane domains to investigate the importance of lipid signalling.
Courtesy of Dominik Erhart from the TraPPs project coordinated by Matthias Wymann, Basel.

Contents

Foreword

The aim of the EUROCORES Programme "Membrane Architecture and Dynamics" (EuroMEMBRANE) was to answer long-standing questions in membrane biology using cutting-edge technologies. Such technologies addressed functional problems in a quantitative manner bringing together experimental tools with theoretical approaches. There was a special emphasis on lipid-lipid and (glyco) lipid-protein interactions in the plane of the membrane in health and disease. Using various model organisms allowed cross-species comparison and brought an evolutionary perspective to biomembrane studies. This type of research required a strong interdisciplinary collaboration covering biological, chemical, physical and computational aspects of membranology over a broad dynamic range of time and length.

Recent technical developments in lipidomics, proteomics and membrane protein structure determination have sparked a new wave of interest in this field. The famous Singer and Nicholson model of a freely mixing 2-dimensional liquid has now been replaced by a more detailed model that recognises additional levels of dynamic organisation both across the lipid bilayer (lipid asymmetry) and laterally (membrane microdomains). The new type of modeling has generated the need to know the actual membrane composition and organisation as this reflects the functions of cells and their organelles, transport of membranes, transport across membranes and signalling.

To find out how the membrane lipidome, proteome and glycome can fulfil all the tasks that membranes have is an enormous challenge. This mission was accomplished by integrated, multidisciplinary approaches involving (bio)chemists, cell biologists, physicists and information technologists (among others) working together to overcome the technical and conceptual barriers that confront the field. We are missing the integrated view of membrane structure and dynamics at the molecular level that is needed to understand membrane changes in aging and diseases such as atherosclerosis, Alzheimer's disease, cancer and a range of infections.

In 2008, the European Science Foundation (ESF) and the European Medical Research Councils (EMRC) published a Science Policy Briefing entitled "*Structural Medicine II: The Importance of Lipidomics for Health and Disease*" with the experts of the now completed European Lipidomics Initiative ELIfe, a specific support action of the European Commission (LSSG-CT-2004-013032, 2005-2007), involving Professors Kai Simons and Elina Ikonen, both Project Leaders of EuroMEMBRANE Collaborative Research Projects[1]. The briefing had concluded that "a better understanding of the lipids in the whole human organism" was required "to further develop diagnostic tools, preventive medicines and therapeutic drugs". With this objective in mind, ESF-EMRC had recommended:

1) Investing in training and research programmes aimed at training biomedical scientists in lipid-related fields.
2) Investing in further development of enabling technologies for lipidomics, while establishing and maintaining strong links between technology developers and the lipid scientific community.
3) Strongly coordinating the interdisciplinary research effort in Europe to understand lipid function with respect to their roles in health and disease with the goal of harmonising lipidomics practices within the European Union.

1. www.lipidomics.net

4) Integrating lipidomics-related databases by supporting initiatives aimed at their communication with other databases worldwide in order to allow a holistic interpretation of the lipid data in the context of health and disease.

No doubt that EuroMEMBRANE has highly contributed to achieving some of these challenging goals. Other research projects and initiatives will also help reach these aims such as the Swiss "LipidX – Systems Biology of Biomembranes"[2] (Principal Investigator: Gisou van der Goot, also EuroMEMBRANE Principal Investigator) or the LipidomicNet European-based initiative[3] that works closely with the US Lipid MAPS Structure Database (LMSD)[4] in order to standardise issues such as the lipids nomenclature.

In the course of its activities, from 2009 to 2012, EuroMEMBRANE consisted of 6 Collaborative Research Projects covering different research areas in cell membrane biology. The major achievements are described in the "scientific highlights" sections of this comprehensive report which also highlights the potential for future developments of research in a field of paramount importance, considering the potential medical applications. A special thank you goes to the 6 Project Leaders and to all the scientists involved in EuroMEMBRANE for their high-level contribution and commitment to this EUROCORES Programme.

Dr Maria Manuela Nogueira
ESF Senior Science Officer in charge of the EuroMEMBRANE Programme and of the Scientific Review Group for Biomedical Sciences

Dr Paola Campus
ESF Senior Science Officer in charge of the Scientific Review Group for Life, Earth and Environmental Sciences

Ms Anne Guehl
ESF EuroMEMBRANE Administrative Coordinator, Process Streamlining and Quality Control Coordinator, Science Service Centre

2. http://www.lipidx.org/
3. LipidomicNet ("Lipid droplets as dynamic organelles of fat deposition and release: Translational research towards human disease") is a FP7 project: http://www.bioinf.med.uni-goettingen.de/projects/lipidomicnet
4. http://www.lipidmaps.org/data/structure/

1.
Governing Bodies

1.1 Management Committee

The Management Committee has the overall responsibility for the EUROCORES Programme.

Dr Remko Achten
Netherlands Organisation for Scientific Research (NWO), The Netherlands

Dr Anna D'Amato
National Research Council (CNR), Italy

Dr Milojka Gindl
Austrian Science Fund (FWF), Austria

Ms Blanka Javorova
Czech Science Foundation (GAČR), Czech Republic

Dr Hannele Lahtinen
Academy of Finland (AKA), Finland

Dr Teresa Ottinger
Swedish Research Council (VR), Sweden

Dr Nikolai Raffler
German Research Foundation (DFG), Germany

Dr Jacob E. Wang
Research Council of Norway (RCN), Norway

Dr Aysim Yilmaz
Swiss National Science Foundation (SNF), Switzerland

1.2 Scientific Committee

The Scientific Committee is made of the 6 Euro-MEMBRANE Project Leaders.

Professor Volker Haucke
Leibniz Institut für Molekulare Pharmakologie and Freie Universität Berlin, Germany

Professor Elina Ikonen
Institute of Biomedicine, University of Helsinki, Finland

Professor Paavo Kinnunen
Institute of Biomedicine, University of Helsinki, Finland

Professor Walter Nickel
Heidelberg University Biochemistry Center, Germany

Professor Kai Simons
Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Professor Matthias Wymann
Institute of Biochemistry and Genetics, University of Basel, Switzerland

1.3 International Review Panel

The independent Review Panel, formed of leading international experts in the field, oversees the selection of projects and the scientific evaluation of the Programme.

Professor John J.M. Bergeron
Department of Anatomy and Cell Biology, Mc Gill University, Montreal, Canada

Professor Oliver T. Fackler
Hygiene Institut des Universitätsklinikums Heidelberg, Germany

Professor Hans-Joachim Galla
Universität Münster, Germany

Professor Michael M. Kozlov
Tel Aviv University, Israel

Professor Joen Luirink
Vrije Universiteit, Department Molecular Microbiology and Pharmacology, Amsterdam, The Netherlands

Professor Margaret Scott Robinson
University of Cambridge, United Kingdom

Professor Shankar Subramaniam
University of California at San Diego, United States

Professor Anders Tengholm
Uppsala University, Sweden

Professor Winfried Weissenhorn
University Joseph Fourier, Grenoble, France

Professor Stephen White
University of California, Irvine, United States

Professor Fred Wouters
Georg-August University Göttingen, Germany

1.4 Funding Organisations

FWF Der Wissenschaftsfonds.

Austria
Austrian Science Fund (FWF)

GAČR Czech Science Foundation

Czech Republic
Czech Science Foundation (GAČR)

ACADEMY OF FINLAND

Finland
The Academy of Finland (AKA)

DFG Deutsche Forschungsgemeinschaft

Germany
German Research Foundation (DFG)

Consiglio Nazionale delle Ricerche

Italy
National Research Council (CNR)

NWO Nederlandse Organisatie voor Wetenschappelijk Onderzoek

The Netherlands
Netherlands Organisation for Scientific Research (NWO)

The Research Council of Norway

Norway
The Research Council of Norway (RCN)

Vetenskapsrådet

Sweden
Swedish Research Council (VR)

FNSNF Swiss National Science Foundation

Switzerland
Swiss National Science Foundation (SNF)

1.5 **Support Team at the ESF**

Dr Arja Kallio
Head of the Life, Earth and Environmental Sciences (LESC) Unit

Dr Paul Egerton
Head of the Life, Earth and Environmental Sciences (LESC) Unit, Head of the Life, Earth, Environmental and Polar Sciences (LEEPS) Unit

Dr Paola Campus
Acting Head of the Life, Earth, Environmental and Polar Sciences (LEEPS) Unit, Senior Science Officer in charge of the Scientific Review Group for Life, Earth and Environmental Sciences (SRG-LEE).

EuroMEMBRANE Science Officers:

Dr Astrid Lunkes
Mr Paul Beckers
Dr Maria Manuela Nogueira

EuroMEMBRANE Administrative Coordinators:

Ms Jackie McLelland
Ms Cindy Régnier
Ms Nicole Stirnberg
Ms Céline Seewald
Ms Eléonore Piémont
Ms Anne Guehl

2. Description of the EuroMEMBRANE Programme

2.1 Rationale and Objectives

How a layer of oil 5 nm thin makes the difference between life and death is simply amazing. The physical laws that govern the behaviour of cellular membranes and their component lipids and proteins are often counterintuitive, especially when coupled with the often bewildering variety of lipids and proteins found in any particular membrane. Recent technical developments in lipidomics, proteomics and membrane protein structure determination have, however, sparked a new wave of interest in this field. The integrated view of membrane structure and dynamics at the molecular level is missing: it is needed to understand membrane changes in aging and diseases such as atherosclerosis, Alzheimer's disease, cancer and a range of infections.

Scientific Goal

The aim of EuroMEMBRANE is to answer long-standing questions in membrane biology that will address functional problems in a quantitative manner by bringing together experimental tools with theoretical approaches. The emphasis will be put on lipid-lipid and (glyco)lipid-protein interactions in healthy or pathologic membranes by using various model organisms to allow cross-species comparison and bring an evolutionary perspective to biomembrane studies. This type of research requires a strong interdisciplinary collaboration that covers biological, chemical, physical and computational aspects of membranology over a broad dynamic range of time and length.

To address the objectives outlined above, the Programme will develop and use cutting-edge applications of technologies, including but not limited to:

- Lipidomics, Proteomics, Glycomics
- Imaging Techniques
- Chemical Biology
- Bioinformatics and Computational Modelling

The latest technology achievements will be employed to tackle long-standing problems in membrane biology in areas such as:

- Membrane and Organelle Biogenesis, Membrane Homeostasis
- Signal Transmission across a Membrane (but not further)
- Non-classical Secretion, Transmembrane Movement of Proteins and Lipids
- Membrane Trafficking
- Cytoskeleton-Membrane Interactions and Lateral Heterogeneity
- Membrane-Pathogen Interactions
- Cell Adhesion and Cell Locomotion
- Lipid Modulation of Membrane Protein Function

The Programme aims at delivering:

- New experimental and computational methods and standards for biological membrane research.
- New biological knowledge on membrane architecture and dynamics that would contribute to a better understanding of membrane functions in the normal and pathologic state.

2.2 List of Projects

The titles and acronyms of the 6 Collaborative Research Projects (CRPs) are the following:

- Molecular Level Physiology and Pathology of Oxidised Phospholipids (OXPL)
- Spatio-temporal Organisation of the Synaptic Membrane for Synaptic Vesicle Protein Recycling (SYNAPSE)
- Tracking of Phosphoinositide Pools – key signalling components in cell migration and polarisation (TraPPs)
- Unconventional Protein Secretion (UPS)
- Lipid-protein Interactions in Membrane Organisation (LIPIDPROD)
- Molecular Determinants of Sterol-Sphingolipid-Protein Interactions in Living Cells and Organisms (Lipid Specific)

More details on each project can be found in Section 3.

2.3 EUROCORES Quality Assurance

2.3.1 Theme Selection

The Theme "How cells shape and utilize their membranes" (Acronym: EuroMEMBRANE) was submitted to the EUROCORES Call for Themes on 22 May 2007 by Professor Gerrit van Meer, Utrecht University, the Netherlands.

2.3.2 Project Selection

Facts and Figures

- **Deadline for Applications:** 22 May 2008

Funded Collaborative Research Projects (CRPs): **6 CRPs** consisting of 29 Individual Projects in **9** different countries, working in close collaboration with 10 Associated Partners from 7 different countries.

- **Duration of Programme:** 2009-2012
- **Budget for Research:** 7.4 Million Euros

The peer review of the CRP proposals in a EUROCORES Programme like EuroMEMBRANE is a multi-stage process, including the establishment of an **international and independent Review Panel** (RP). In response to an open call for proposals, **outline proposals** are submitted by a team of applicants (minimum 3 from 3 different countries). At that stage, the RP is responsible for the sifting of outline proposals prior to the invitation of full proposals. At the **full proposal stage**, each proposal is sent for **written external assessments** to at least 3 referees. Applicants are given the opportunity to reply to the anonymous referee reports (rebuttal).

Written referees' assessments and replies by applicants are then considered by the RP with scientific quality being the main selection criterion. The RP makes recommendations for funding of CRPs with prioritisation, which ESF communicates to the EUROCORES funding organisations.

36 outline proposals were submitted to the call launched in 2008, out of which 17 were invited to proceed to the full proposal stage. As described in the previous section and following the international peer review procedure, 6 CRPs were selected and launched in 2009 with the first kick-off meeting taking place on 16 June 2009 in Strasbourg, France.

2.3.3 Management Committee

When the call for proposals was published, the EuroMEMBRANE Management Committee (MC) was established (see page 5).

- The MC has overall responsibility for the EUROCORES Programme within the guidelines of the EUROCORES Scheme;
- The MC can request expert advice from the EUROCORES Scientific Committee, Review Panel or any other *ad-hoc* advisory group;
- Members support the EUROCORES review process by nominating potential Review Panel and external expert referees on behalf of their funding organisation;
- Each MC member is responsible for liaising with their funding organisation, including supervision of the funding process for EUROCORES projects within their organisation;
- Members may attend meetings of the EUROCORES Programme as observers.

2.3.4 Mid-Term and Final Evaluations

Each EUROCORES Programme undergoes 2 comprehensive reviews to evaluate their progress at the mid- and final stages. The aim is to assess scientific cooperation and interactions among the investigators and examine the merits of the EUROCORES Programme. At the mid-term stage, the potential of the Programme should be assessed while the lessons to be learned for potential follow-up initiatives should be evaluated at the final stage.

Assessment criteria include:

- Novelty/Originality
- Multidisciplinary Research

- Collaborative Research
- European added value
- Relevance to the Call

The EuroMEMBRANE Review Panel Members produced a consensus statement at the occasion of the final evaluation. See page 34 as a conclusion to this final report.

2.3.5 **EUROCORES Acknowledgements**

All publications, posters, websites and other dissemination outputs are required to be clearly identified as being Programme-funded or co-funded. For EuroMEMBRANE the acknowledgement was:

The aim of the European Collaborative Research (EUROCORES) Scheme is to enable researchers in different European countries to develop collaboration and scientific synergy in areas where European scale and scope are required to reach the critical mass necessary for top class science in a global context.

The scheme provides a flexible framework which allows national basic research funding and performing organisations to join forces to support excellent European research in and across all scientific areas.

The European Science Foundation (ESF) provides scientific coordination and support for networking activities of funded scientists currently through the EC FP6 Programme, under contract no. ERAS-CT-2003-980409. Research funding is provided by participating organisations. EuroMEMBRANE is managed by the Life, Earth and Environmental Sciences (LESC) Unit at the ESF.

3.
Highlights of the Collaborative Research Projects

3.1 Molecular Level Physiology and Pathology of Oxidised Phospholipids (OXPL)

Project Leader:
Professor Paavo Kinnunen
University of Helsinki, Finland

Principal Investigators:
Professor Albin Hermetter
Graz University of Technology, Austria
Professor Martin Hof
Academy of Sciences of the Czech Republic, Prague, Czech Republic
Professor Thorsten Hugel
Technical University Munich, Garching, Germany
Professor Pavel Jungwirth
Academy of Sciences of the Czech Republic, Prague, Czech Republic
Dr Gerhard Schütz
Johannes Kepler University Linz, Austria

Associated Partners:
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Umeå University, Sweden
Dr Himanshu Khandelia
University of Southern Denmark (SDU), Odense, Denmark
Dr Francesco Megli
University of Bari, Italy
Dr Ingela Parmryd
Uppsala University, Sweden
Dr Corinne Spickett
University of Strathclyde, Glasgow, United Kingdom

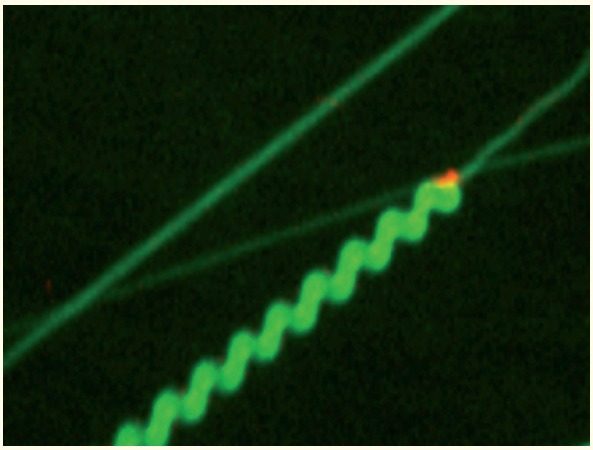

Figure 1.
Formation of a amylin fibril surrounded by a helical lipid nanotube (green) after the addition of amylin on a supported lipid bilayer containing a oxidized phosphocholine derivative PazePC. Courtesy of Kibron Inc.

Funding Organisations:
Austria: *Austrian Science Fund (FWF)*
Czech Republic: *Czech Science Foundation (GAČR)*
Finland: *Academy of Finland (AKA)*
Germany: *German Research Foundation (DFG)*

Scientific background and objectives

The OXPL consortium represents a highly focused, integrated interdisciplinary approach of European research laboratories already active in this now rapidly emerging area in biological chemistry, together with additional skills brought into the project by groups representing state-of-the-art expertise in method and high-resolution instrumentation development for the characterisation of biomolecules, their interactions in biomembranes and localisation in cells. More specifically, this project combines leading-edge expertise on membrane biophysics,

from modern molecular dynamics simulations to a range of experimental techniques (ellipsometry, Langmuir troughs, FCS, AFM, NMR), all the way to protein folding, molecular cell biology, imaging, chemical biology and lipidomics analyses. The project will pave the way to the development of improved diagnostics, therapies and preventive measures to combat the above diseases, and will take European research to the leading edge in this rapidly emerging and important area.

Scientific highlights

One of the major tasks in OXPL was to establish the molecular level impact of oxidized phospholipids on the biophysical characteristics of lipid bilayers. This task was pursued in several groups and involved efficient collaborations. The following effects could be established: 1) decrease in lipid order, 2) lowering of phase transition temperatures, 3) lateral expansion and thinning of the bilayer, 4) alterations of bilayer hydration profiles, 5) increased lipid mobility, 6) augmented flip-flop, 7) influence on the lateral phase organisation and 8) promotion of water defects and, under extreme conditions, disintegration of the bilayer. All the above findings were substantiated by experiments and simulations, involving close collaborations both within and outside the OXPL CRP.

The augmented flip-flop may bear special importance, as this finding implies that there is a need for a paradigm shift. More specifically, in life sciences there is tendency to explain all processes in terms of reflecting the action of a specific enzyme. In apoptosis, for instance, there is rapid loss of phospholipid asymmetry, with phosphatidylserine (PS) translocating across the plasma membrane and becoming exposed on the outer surface of apoptotic cells. This process has been assumed to reflect the action of scramblase protein, an enzyme catalysing the transfer of PS across the bilayer. Our data show that simple introduction of oxidized phosphatidylcholine is sufficient to increase the rate of PS flip-flop. Accordingly, change in specific biophysical properties imposed by phospholipid oxidation would suffice, alleviating the need to involve putative membrane proteins.

Using single molecule tracking Schütz *et al.* detected a transient immobilisation of fluorescently labelled oxidized phospholipids (PGPE) at highly curved membrane regions such as clathrin-coated pits and caveolae. They further investigated the behaviour of fluorescent PGPE analogues in model membranes. Compared to a standard phospholipid, the PGPE-analogue yielded significantly higher mobility; the difference levelled off with increasing cholesterol content. By molecular dynamics simulations they could show that the PGPE headgroups are located at the outside of the bulk membrane headgroups; increasing cholesterol concentrations shift PGPE towards the bilayer center and further increases the interactions with the bulk membrane. Along similar lines using atomic force microscopy (AFM), Hugel in collaboration with Hermetter and Jungwirth determined the interaction energy between a single lipid and a supported lipid bilayer for POPE and PGPE. For POPE the interaction force is around 30 pN, while the interaction with the PGPE-OxPL was below the noise limit of the instrument (around 10pN). Therefore, they could tentatively conjecture that the interaction of OXPL with other lipids in a lipid bilayer is strongly reduced – almost down to the thermal energy. These results are consistent with the huge difference in critical micelle concentration for POPE (~nM) and PGPE (~μM). Importantly, the addition of oxidized PC results in a reduction of lipid membrane elastic parameters namely, membrane stability, bilayer thickness, Young's modulus, area stretch modulus and bending stiffness.

Schütz *et al.* investigated the effect of oxidized phospholipids on the integrity of membrane rafts. Using single molecule brightness analysis they found substantial reduction in the homoassociation of raft proteins upon treatment of cells with POVPC. The effect is remarkable, as it may help to understand the mechanisms of raft cohesion. Simultaneously, Kinnunen *et al.* showed that oxidized phosphatidylcholine promoted the stability of liquid ordered phase formed by cholesterol and sphingomyelin, demonstrating that lipid oxidation has profound impact on membrane dynamic organisation. Parmryd *et al.* are studying the effect of POVPC and PGPC on T cell signalling and membrane order in collaboration with Hermetter *et al.* They have found that the oxidised lipids activate T cells through the ERK signalling pathway.

Notably, the Kinnunen group demonstrated that oxidized PC could be shown to promote amyloid formation by pathology related gelsolin fragment as well as by a protein, phospholipase A2. For the latter this resulted in transient activation of this enzyme. This process is discussed in more detail in a special issue of *Biochimica et Biophysica Acta*, and is being investigated further by mass spectrometry analysis in collaboration with Spickett. The special issue also describes the current status of protein reactions with aldehydophospholipids, reviewed by Stemmer and Hermetter.

Somewhat along similar lines, the finding by Gröbner *et al.* of the Bax protein modulation by oxidized PC in mitochondria could be ground-breaking.

Hermetter *et al.* study the interaction of proteins with short-chain oxidized phospholipids in cultured macrophages. They have cloned expression vectors for fusion proteins consisting of selected candidate proteins[1] and RFP or GFP, and successfully expressed the fluorescent polypeptides in RAW macrophages. The spatial proximity of these proteins and fluorescently labelled oxidized phospholipids is currently being investigated in collaboration with the Hof and Schütz laboratories. Expression of candidate proteins could be efficiently silenced with siRNA for loss of function studies to determine their functional contribution to lipid toxicity. Alternatively, protein activity was "silenced" by chemical inhibitors. This approach enabled to identify acid sphingomyelinase as a functional target of aldehydophospholipids in macrophages which is causally involved in lipid-induced cell death[2].

On top of answering the above biophysical questions, the original proposal also contained several methodological deliverables. Hof *et al.* completed the set up of a dynamic saturation optical microscope, the implementation of raster image correlation spectroscopy, a new fluorescence approach allowing for the first time analysis of both dynamics and size of raft nanodomains being in the order of 5 to 20 nm, a new approach for real-time monitoring the transbilayer movement, and the presentation of a new version of 2-focus fluorescence correlation spectroscopy. Jungwirth *et al.* established atomistic-level force field (i.e. interaction potentials) for OXPL and robust simulation protocol for stable MD simulations of aqueous oxidized membranes in an aqueous environment.

European added value

The OXPL CRP brought a significant number of biophysicists together and provided a strong motivation for e.g. physicochemists to study lipids and membranes. Accordingly, the impact of the OXPL will remain visible for several years to come.

1. Stemmer U, Ramprecht C, Zenzmaier E, Stojčić B, Rechberger G, Kollroser M, Hermetter A. Uptake and protein targeting of fluorescent oxidized phospholipids in cultured RAW 264.7 macrophages. *Biochim. Biophys. Acta.* 1821 (4): 706-718 (2012)
2. Stemmer U, Dunai ZA, Koller D, Pürstinger G, Zenzmaier E, Deigner HP, Aflaki E, Kratky D, Hermetter A. Toxicity of oxidized phospholipids in cultured macrophages. *Lipids in Health and Disease.* 11: 110 (2012); Stemmer U, Ramprecht C, Zenzmaier E, Stojčić B, Rechberger G, Kollroser M, Hermetter A. Uptake and protein targeting of fluorescent oxidized phospholipids in cultured RAW 264.7 macrophages. *Biochim Biophys Acta.* 1821 (4): 706-718 (2012)

Elucidation of the molecular mechanisms involving oxidized phospholipids in the pathogenesis of protein misfolding/aggregation diseases will definitely open new areas of activity, most notably to develop preventive measures to combat these ailments, developing globally amongst the aging population. A joint activity involving basic studies such as the OXPL CRP and clinicians investigating Alzheimer's, Parkinson's, Age-related Macular Degeneration and type 2 diabetes could be expected to have potential for major impact on European healthcare, allowing to educate risk groups about proper nutritional practises. In this case this is a particularly realistic approach, as the fatty acid composition of cellular membrane lipids is perhaps the most easily modifiable chemical environment in our cells, which can be readily influenced by the diet. Studies along the above lines are now being initiated by Kinnunen *et al.* in Finland.

Overall self-assessment on the accomplishments of the CRP

Significant deviations did not happen. The fact that Gröbner and Parmryd did not receive funding from the Swedish Research Council had a serious impact on the Programme. Taking into account the extremely competitive nature of this call, the level of funding from the Academy of Finland was also insufficient to enable research visits to the extent planned. The UK participant Spickett was also not funded but yet could contribute in a collaboration which is still ongoing with the laboratory of Kinnunen, identifying the reaction products of aldehydophospholipids with a protein *in vitro*.

Selected publications

Volinsky RR, Cwiklik L, Jurkiewicz P, Hof M, Jungwirth P, Kinnunen PKJ. Oxidized Phosphatidylcholines Facilitate Phospholipid Flip-Flop in Liposomes. *Biophysical Journal.* 101 (6): 1376-1384 (2011)

Volinsky RR, Paananen R, Kinnunen PKJ. Oxidized Phosphatidyl-cholines Promote Phase Separation of Cholesterol-Sphingomyelin Domains. *Biophysical Journal* 103: 247-254 (2012)

Wallgren M, Beranova L, Dat Pham Q, Khanh L, Lidman M, Procek J, Cyprych K, Kinnunen P, Hof M, Gröbner G. Impact of oxidized phospholipids on the structural and dynamic organisation of phospholipid membranes: a combined DSC and solid state NMR study. *Faraday Discuss.* 161: 499-513 (2013)

Plochberger B, Stockner T, Chiantia S,

Brameshuber M, Weghuber J, Hermetter A, Schwille P, Schütz GJ. Cholesterol slows down the lateral mobility of an oxidized phospholipid in a supported lipid bilayer. *Langmuir* 26: 17322-17329 (2010)

Kinnunen P, Hermetter A, Spickett CM (guest editors). Oxidized Phospholipids – Their Properties and Interactions with Proteins. Special Issue of *Biochim. Biophys. Acta* 1818: 2373-2475 (2012)

3.2 Spatio-temporal Organisation of the Synaptic Membrane for Synaptic Vesicle Protein Recycling (SYNAPSE)

Project Leader:

Professor Volker Haucke

Leibniz Institut für Molekulare Pharmakologie and Freie Universität Berlin, Germany

Principal Investigators:

Professor Pekka Hänninen

University of Turku, Finland

Dr Jürgen Klingauf

University of Münster, Germany

Professor Oleg Shupliakov

Karolinska Institute, Stockholm, Sweden

Funding Organisations:

Finland: *Academy of Finland (AKA)*

Germany: *German Research Foundation (DFG)*

Sweden: *Swedish Research Council (VR)*

Scientific background and objectives

The presynaptic terminal contains clusters of synaptic vesicles (SV), key organelles of chemical neurotransmission. Proteomics data indicate that these vesicles comprise distinct sets of proteins and lipids present in defined stoichiometries, which must be maintained during repetitive rounds of exo- and endocytosis. How this precise sorting of the synaptic vesicle membrane proteins is accomplished molecularly is not well understood. How the spatio-temporal dynamics of endocytic proteins at the nerve terminal are controlled is largely unknown but likely involves membrane-associated multidomain scaffolding proteins organising the periactive zone. This project aimed to tackle these problems and gain unprecedented insights into the spatio-temporal organisation of the synaptic membrane for clathrin-mediated synaptic vesicles protein recycling.

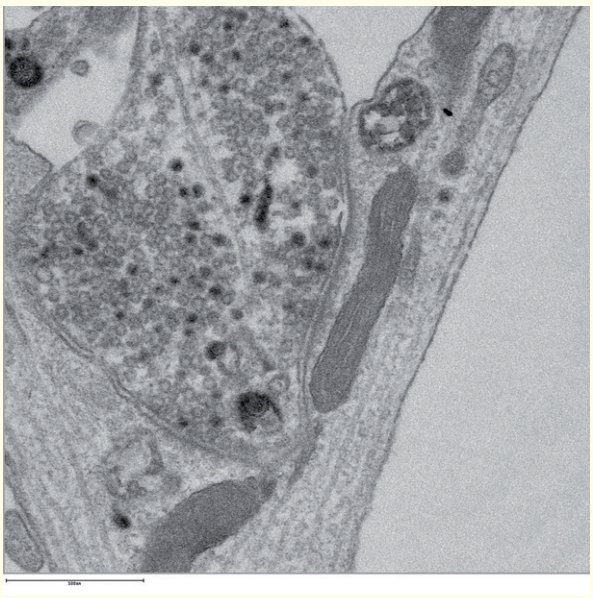

Figure 2.
Electron micrograph of synapses between hippocampal neurons in culture. Presynaptic terminals are filled with synaptic vesicles. Recycling vesicles and endosomal structures were labeled with horseradish peroxidase followed by photoconversion. Scale bar, 500 nm.
Courtesy of Volker Haucke (SYNAPSE Project Leader) and Oleg Shupliakov (SYNAPSE Principal Investigator)

Scientific highlights

The recent development of the first known small molecular inhibitors of clathrin function is the scientific highlight of this CRP.

Within the endocytic network clathrin functions as a central organising platform for coated pit assembly and dissociation. Despite the extensive knowledge about the molecular architecture of endocytic proteins, the role of the clathrin terminal domain (TD) in clathrin-mediated endocytosis remains unknown. A chemical biology approach was taken to tackle this question. Two novel compounds, named pitstops that selectively block endocytic ligand association with the clathrin TD as confirmed by X-ray crystallography, were designed and synthesised. Pitstop-induced inhibition of clathrin TD function acutely interferes with receptor-mediated endocytosis, entry of HIV, and synaptic vesicle recycling. Endocytosis inhibition is caused by a dramatic increase in the lifetimes of clathrin coat components including FCHo, clathrin and dynamin. These data unravel a crucial unexpected role for the clathrin TD in regulating coated pit dynamics. Moreover, pitstops provide new tools to address clathrin function in cell physiology with potential applications as inhibitors of virus and pathogen entry and as modulators of cell signalling.

Currently, pitstops are being used to unravel the role of clathrin in SV exo-endocytosis at central synapses under different stimulation paradigms using

advanced optical imaging tools and novel fluorescent probes in cultured neurons and 3D-electron microscopy using the lamprey giant synapse (collaboration between Haucke, Klingauf and Shupliakov). Surprisingly and unexpectedly, using pitstops in cultured hippocampal neurons, it was found that impaired endocytosis leads to a novel form of fast short-term depression, which is not related to insufficient vesicle supply at the presynaptic bouton but is a result of slow clearance of vesicular components from the release site. This finding implies an important role of endocytic proteins for sustained synaptic transmission at high rates beyond their well-established roles in early and late steps of endocytosis.

A second major achievement that has emanated from a cross-CRP collaboration between the 2 Principal Investigators Haucke and Schultz (TraPPs) has resulted in the identification of phosphatidylinositol 3,4-bisphosphate [$PI(3,4)P_2$] as a novel lipid that spatio-temporally controls clathrin-mediated endocytosis in general. The formation of $PI(3,4)P_2$ by class II phosphatidylinositol 3-kinase C2α (PI3K C2α) spatio-temporally controls clathrin-mediated endocytosis. Depletion of $PI(3,4)P_2$ or PI3K C2α impairs the maturation of clathrin-coated pits at a late stage preceding fission. Timed formation of $PI(3,4)P_2$ by PI3K C2α is required for the selective enrichment of the BAR domain protein SNX9 at late-stage endocytic intermediates, in accordance with mathematical modelling. These findings provide a mechanistic framework for the role of $PI(3,4)P_2$ in endocytosis and unravel a novel discrete function of $PI(3,4)P_2$ in a central cell physiological process.

Lastly, Hänninen *et al.* have established a dual-modal simultaneous imaging system that combines AFM and STED microscopy. The set-up provides information on the topological and mechanical properties at the atomic scale by AFM, overlaid precisely onto super-resolution STED fluorescence images, which will be applied to induce neuronal signalling on demand by the AFM cantilever while conducting live STED imaging. This work has not been published yet.

European added value

As a follow-up Haucke *et al.* have framed an application in the context of the ERA-NET Neuron call 2012. The proposed project with consortium members from Germany, Belgium and Canada directly benefits from results obtained within the EuroMEMBRANE programme and deals with the "Spatio-temporal control of exo-endocytosis in the regulation of synaptic function in health and disease". The aim is to better understand presynaptic dysfunction which is linked at the cellular and molecular levels to neurodegenerative disorders including Morbus Parkinson's, Huntington's and Alzheimer's diseases, among others. The proposed research is based on the hypothesis that SV cycling is spatio-temporally controlled by the regulation of GTPase modules comprising Dynamin, Cdc42, RalA, Arf6 and Rab35 via a network of interacting GAPs and GEFs that associate directly or indirectly with multidomain scaffold proteins at synapses. Neuronal dysfunction in this model arises from subtle defects within this regulatory GTPase network that are difficult to discern by conventional methodologies, necessitating the development of novel acute perturbation and innovative imaging techniques. The proposed project will fill-in this gap by developing cutting-edge technologies including optogenetic and chemical biology approaches for acute functional intervention, genetics, as well as super-resolution and vital optical imaging of subsynaptic structures tailored to dissect presynaptic vesicle cycling at unprecedented resolution. The proposed international project had passed the initial stage but eventually did not receive funding in spite of excellent reviews.

Research conducted within EuroMEMBRANE has sparked via a cross-CRP collaboration between Schultz and Haucke about the investigation of the role of new species of phosphoinositides in endocytosis. PI(3,4)P2 formation via the clathrin-associated PI3-kinase type C2alpha spatiotemporally controls clathrin-mediated endocytosis by facilitating the recruitment of the PX-BAR domain protein SNX9 at a late stage preceding dynamin-based vesicle fission. Research on PI(3,4)P2 has thus become a major interest in Haucke's lab. These findings will also serve as a basis for an application for funding within a planned DFG priority programme pertaining to membrane traffic in health and disease (led by Thomas Seufferlein, University of Ulm, Germany).

As a second spin-off from EuroMEMBRANE, several Principal Investigators from SYNAPSE fueled by technological developments by Hänninen have developed methodology for multi-color super-resolution imaging including STED (Haucke, Hänninen, Shupliakov), PALM (Klingauf), and dSTORM (Haucke) microscopy. These have already taken centre stage in daily research and have become part of applications for funding within a variety of national and international programmes.

Overall self-assessment on the accomplishments of the CRP

The CRP has reached all its major objectives given the time frame of the programme including the publication of major breakthroughs in leading international journals. The collaboration between all Principal Investigators has been particularly fruitful regarding pre-synaptic organisation, whereas technology development and publications emanating from these have lagged somewhat behind.

The specific goals were originally:

1) *Visualisation and detailed characterisation of key components of the periactive zone by advanced fluorescent labelling strategies in combination with high-resolution imaging techniques such as TIRF, FPALM, STED and correlative light and electron microscopy, as well as electron tomography.*
These have been largely accomplished though not all results have been published yet.

2) *Manipulation and perturbation of select interactions between scaffolding, endocytic proteins and their partners including inositol phospholipids within the periactive zone by genetic, biochemical and chemical tools in complementary model systems.*
These are ongoing efforts but major sets of data emanating from the analysis of various mouse and fly endocytic protein mutants as well as from acute perturbation experiments have already been published. Moreover, a novel lipid [PI(3,4)P2] has been identified in endocytosis whose relationship to and specific function at synapses will need to be explored in the future.

3) *Integration of data derived from the aforementioned approaches into a simple reaction-diffusion model using deterministic as well as Monte Carlo simulations in order to make precise predictions for certain perturbations and this way guide and stimulate further experiments.*
These have been partly incorporated into ongoing projects pertaining to the diffusion behavior of newly exocytosed SV proteins (Haucke, Klingauf). Also, such models have played a critical role in understanding the function of PI(3,4)P2 in the recruitment of endocytic proteins (Posor *et al.*, submitted) (Haucke). Hence, work along these lines has paved the way for further more detailed theoretical studies.

Selected publications

Kononenko NL, Diril MK, Puchkov D, Kintscher M, Koo SJ, Pfuhl G, Winter Y, Wienisch M, Klingauf J, Breustedt J, Schmitz D, Maritzen T, Haucke V. Compromised fidelity of endocytic synaptic vesicle protein sorting in the absence of stonin 2. *Proc Natl Acad Sci U S A.* 110 (6): E526-E535 (2013)

Hua Y, Sinha R, Thiel C, Schmidt R, Hüve J, Marten H, Hell SW, Egner A, Klingauf J. A readily retrievable pool of synaptic vesicles. *Nat Neurosci.* 14: 833-839 (2011)

Jiao W, Masich S, Franzén O, Shupliakov O. Two pools of vesicles associated with the presynaptic cytosolic projection in Drosophila neuromuscular junctions. *J Struct Biol.* 172: 389-394 (2010)

Winther Å, Jiao W, Vorontsova O, Rees KA, Koh TW, Sopova E, Schulze KL, Bellen H, Shupliakov O. The dynamin-binding domains of Dap160/Intersectin affect bulk membrane retrieval in synapses. *J. Cell Sci.* (2013) Jan 15 [Epub ahead of print]

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Rao Y, Ma Q, Vahedi-Faridi A, Sundborger A, Pechstein A, Puchkov D, Luo L, Shupliakov O, Saenger W, Haucke V. Molecular basis for SH3 domain regulation of F-BAR-mediated membrane deformation. *Proc. Natl. Acad. Sci. USA.* 107: 8213-8218 (2010)

von Kleist L, Stahlschmidt W, Bulut H, Gromova K, Puchkov D, Robertson M, MacGregor KA, Tomlin N, Pechstein A, Chau N, Chircop M, Sakoff J, von Kries J, Saenger W, Kräusslich H-G, Shupliakov O, Robinson P, McCluskey A, Haucke V. Essential role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* 146:471-84 (2011)

Posor Y, Eichhorn-Gruenig M, Puchkov D, Schöneberg J, Ullrich A, Lampe A, Müller R, Zarbakhsh S, Schultz C, Schmoranzer J, Noé F, Haucke V. Spatiotemporal Control of Endocytosis by Phosphatidylinositol 3,4-Bisphosphate. Under revision at *Nature* (2013)

Hua Y, Woehler A, Kahms M, Haucke V, Neher

E, Klingauf J. Blocking endocytosis enhances short-term synaptic depression under conditions of normal availability of vesicles. Under revision at *Neuron* (2013)

3.3 Tracking of Phosphoinositide Pools – key signalling components in cell migration and polarisation (TraPPs)

Project Leader:
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Principal Investigators:
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Professor Karl-Eric Magnusson
University of Linköping, Sweden
Professor Carsten Schultz
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Associated Partners:
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University of Basel, Switzerland
Professor B. Christoffer Lagerholm
University of Southern Denmark, Odense, Denmark
Professor Markus R. Wenk
National University of Singapore

Funding Organisations:
Germany: *German Research Foundation (DFG)*
The Netherlands: *Netherlands Organisation for Scientific Research (NWO)*
Norway: *The Research Council of Norway (RCN)*
Sweden: *Swedish Research Council (VR)*
Switzerland: *Swiss National Science Foundation (SNF)*

Scientific background and objectives

Membrane dynamics modulate cell polarity, vesicular trafficking, migration, growth, proliferation, differentiation and more. Of all membrane lipids, phosphoinositides play a central role in these processes. Although a role for the prominent 3-phosphorylated phosphoinositides such as PtdIns(3,4,5)P3 and PtdIns(3)P has been highlighted in physiology and disease, dynamics and localisation of these lipids are still poorly understood. TraPPs will provide a dynamic and refined view of phosphoinositide flux and required lipid modifying enzymes, e.g., PI3Ks, lipases and lipid phosphatases. Lipid-modifying enzymes will be targeted dynamically to distinct cellular locations, and lipid-interacting proteins shall be manipulated to display their free or lipid-bound state. Activation of lipid modifying enzymes will be linked to localised upstream signalling and specific cell responses. Cellular, genetic fly and mouse models will be used to validate the uncovered molecular mechanisms. This project provides the basis for a broader systems biology approach of lipid signalling and will elucidate dynamic cellular processes relevant to cancer and inflammation.

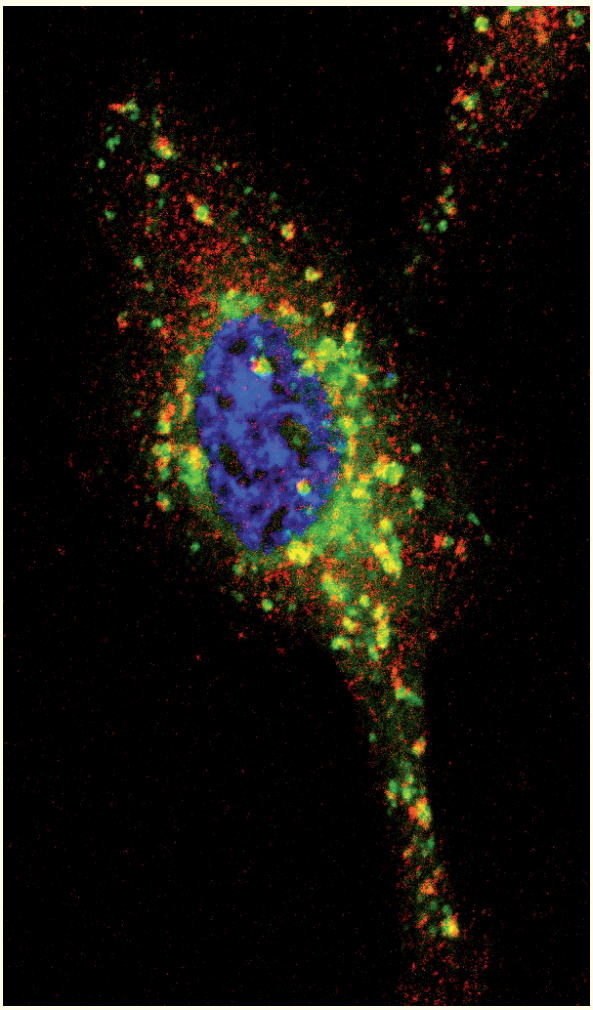

Figure 3.
Membrane organisation has effects on the metabolic state of melanoma cancer cells: depending on the state of endosomal organelles stained in green here (using a eGFP-Rab7 probe), the nutrient sensor target of rapamycin complex 1 (TORC1, shown in red) is delocalised. Phosphoinositides play an important role in the control of the activity of TORC1. This image emerges from a collaboration between the *TraPPs* partners Wymann, Schultz and Wenk (Melone *et al.*), where also cell permeable, phosphoinositides were utilised to stimulate TORC1.
Courtesy of Anna Melone.

Scientific highlights

The development of novel tools to study phosphoinositide (PI) biology is a crucial basis for the progress in improved assessment of PI flux and function. Synthetic organic chemistry approaches have been completed, and major technical developments in imaging of lipids have been achieved. The combination of novel probes, chemical tools, and imaging techniques required major efforts in coordination.

The Schultz group prepared and tested the first membrane-permeant photoactivatable phosphoinositides for life cell applications. These are sophisticated tools for cell biology, which are already used by various groups within TraPPs and other EuroMEMBRANE CRPs. Caged derivatives could be utilised by the Schultz group to show that fatty acid composition of diacylglycerols determined ion channel opening in the plasma membrane. Additionally, membrane-permeant lipids from the Schultz group were used by the Haucke group (CRP SYNAPSE) to demonstrate the contribution of elevated PtdIns(3,4)P2 levels in productive endocytotic processes.

The Wymann group also benefited from the availability of cell-permeable PtdIns(3)P to investigate the role of Vps34 class III PI3K, class II PI3Ks and their products in the activation process of the amino acid induced activation of the TORC1 complex. Surprisingly, Wymann *et al.* found that PtdIns(3)P attenuated TORC1 activity of a TORC1 complex docked on late endosomes, which provides novel insight in the controversial role of Vps34 in the TORC1 activation process.

A set of protein dimerizer tools (xCrASH, Schultz; rapamycin-triggered G-protein subunits, Schultz; HaxS, Wymann) have opened the door to orthogonal and multiplexed approaches in the targeting of lipid-producing enzyme assemblies to selective cellular compartments. Both groups have completed proof-of-concept applications for the respective systems and Wymann has achieved HaXS-mediated activation of a PI3K system.

Gadella developed and/or improved new (single molecule) advanced microscopy methods such as super-resolution PALM – TIRF mode, Number & Brightness (N&B) analysis, line scanning FCS and Quantitative FRAP algorithms to discern molecular complex formation at the plasma membrane. Using these techniques the phospholipid/Ca+ binding protein Annexin A4 was studied and its transition from a monomeric state to the formation of trimers induced by Ca^{2+}-dependent relocation to the plasma membrane was monitored. The analysis of stimulation-dependent interaction of Gαq and downstream effectors, including PLCβ1a, p63RhoGEF and PI3Kα, contradict some published results that claim that Gαq signalling is mediated via a set of invariant, predetermined protein complexes.

With Riezman (Lipid Specific), Wenk has investigated cholesterol-producing yeast strains, which utilise cholesterol instead of ergosterol. In the light of earlier work describing a clear-cut link between sphingolipid and sterol biosynthesis, these novel results provide important insights to processes mediating lipid hemostasis.

Besides uncovering novel roles for class III PI3K and its product PtdIns(3)P in cancer-promoting processes, like cell division, cytokinesis and endosomal sorting, the group of Stenmark has identified novel cancer-associated mutations in PtdIns(3)P-binding proteins. Moreover, Stenmark has provided novel links and mechanisms how PtdIns(3)P proteins can modulate growth factor turnover and activity.

European added value

The TraPPs and EuroMEMBRANE-initiated collaborations will provide a more detailed picture of the function of specific phosphoinositides. The initiative has also triggered developments that will not only boost the understanding of the enzyme cascades modifying head groups of phosphoinositides, but also their defined fatty acid compositions. This, and the possibility to explore specific membrane and sub-membrane targeting of lipid-modifying enzymes, will open a new level of understanding to membrane compartments in constant fluid motion.

TraPPs activities are directly related to:

- a follow-up project on rapamycin-induced enzyme activities with a focus on sphingolipids (Schultz), funded by the EU via an Initial Training Network (ITN).
- Swiss National Science Foundation (SNSF) funding obtained by Wymann and Constable for interdisciplinary and collaborative projects:
  - 2013-2014: SNSF 205320_143699: Covalent tag-chemistry targeting receptor dynamics and signalling. 91 kCHF
  - 2011-2012: SNSF 205320_138302: Chemical Control of Intracellular Signalling Cascades. 60 kCHF
  - 2010-2011: SNSF 205321_126508: Dynamic Protein Dimerization and Membrane Translocation. 219 kCHF
- a Commission for Technology and Innovation (CTI) grant (to Wymann) that was enabled by the presence of a chemical biology activity funded by TraPPs. The theme is related to TraPPs activities

(targeting PI3Ks by small molecules).
– 2012-2013: CTI grant 14032.1 PFLS-LS with PIQUR Therapeutics. Full cost 872 kCHF; CTI federal grant contribution: 581 kCHF

TraPPs-induced contacts of Magnusson have resulted in a connection between the Netherlands and Swedish BioImaging initiatives for the European Strategy Forum on Research Infrastructures (ESFRI) EuroBioImaging network[3]. Magnusson is the mediator between the 2 national initiatives.

Overall self-assessment on the accomplishments of the CRP

The TraPPs members produced more than the anticipated chemical tools, cell-permeable phosphoinositides and probes. The applied chemical strategies have generated a broad repertoire of starting points, and several lines of products required extensive development and tuning. This had 2 effects: there are multiple opportunities for further developments but not all molecules have already been extensively used in biological systems. A broad application of probes, tools and microscopic techniques will be published therefore after the termination of EuroMEMBRANE.

The same applies to collaborative projects, which were very interdisciplinary in nature, and required sometimes cycling between chemistry optimisation and improvement of biological probe and detection systems.

Selected publications

Accepted, in press:

Schultz C, Neef AB, Gadella TWJ, Goedhart J. Imaging lipids in living cells. *Cold Spring Harb Protoc 2010*, pdb.top83 (2010)

Adjobo-Hermans MJW, Crosby KC, Putyrski M, Bhageloe A, van Weeren L, Schultz C, Goedhart J, Gadella Jr. TWJ. PLCβ isoforms differ in their subcellular location and their CT-domain dependent interaction with Gαq. *Cell Signalling*. 25 (1): 255-63 (2013)

Wymann M, Schultz C. The chemical biology of PI 3-kinase. *ChemBioChem.* 13 (14): 2022-2035 (2012)

Wymann MP, Wenk MR. Neutral is not a loss – detection of phosphoinositides beyond the head group. *Nat. Methods*. 8: 219 (2011)

(Re-)submitted:

Melone A, Marone R, Subramanian D, Schultz C, Wymann MP. 3-phosphorylated phosphoinositides are required for TORC1 assembly, but inhibit docked TORC1.

Erhart D, Zimmermann M, Jacques O, Wittwer M, Ernst B, Constable EC, Zvelebil M, Beaufils F, Wymann MP. Chemical Development of Intracellular Protein Heterodimerizers.

3. http://www.eurobioimaging.eu/

3.4 Unconventional Protein Secretion (UPS)

Project Leader:

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Associated Partner:

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Funding Organisations

Germany: *German Research Foundation (DFG)*
The Netherlands: *Netherlands Organisation for Scientific Research (NWO)*
Switzerland: *Swiss National Science Foundation (SNF)*

Scientific background and objectives

Two types of non-classical protein transport to the cell surface of eukaryotic cells have been described, processes that collectively have been termed 'Unconventional Protein Secretion' (UPS). The first type is represented by integral membrane proteins that can reach the cell surface in a Golgi-independent manner. The second type is used by soluble secretory proteins that exit cells without any involvement of the endoplasmic reticulum (ER) and the Golgi system. While integrins are an example for membrane proteins transported by unconventional means, the class of soluble unconventional secretory proteins includes interleukin family members, fibroblast growth factors, macrophage migration inhibitory factor, galectins and acyl-CoA binding protein, macromolecules that are secreted from cells to regulate the immune response, cell growth and differentiation as well as angiogenesis.

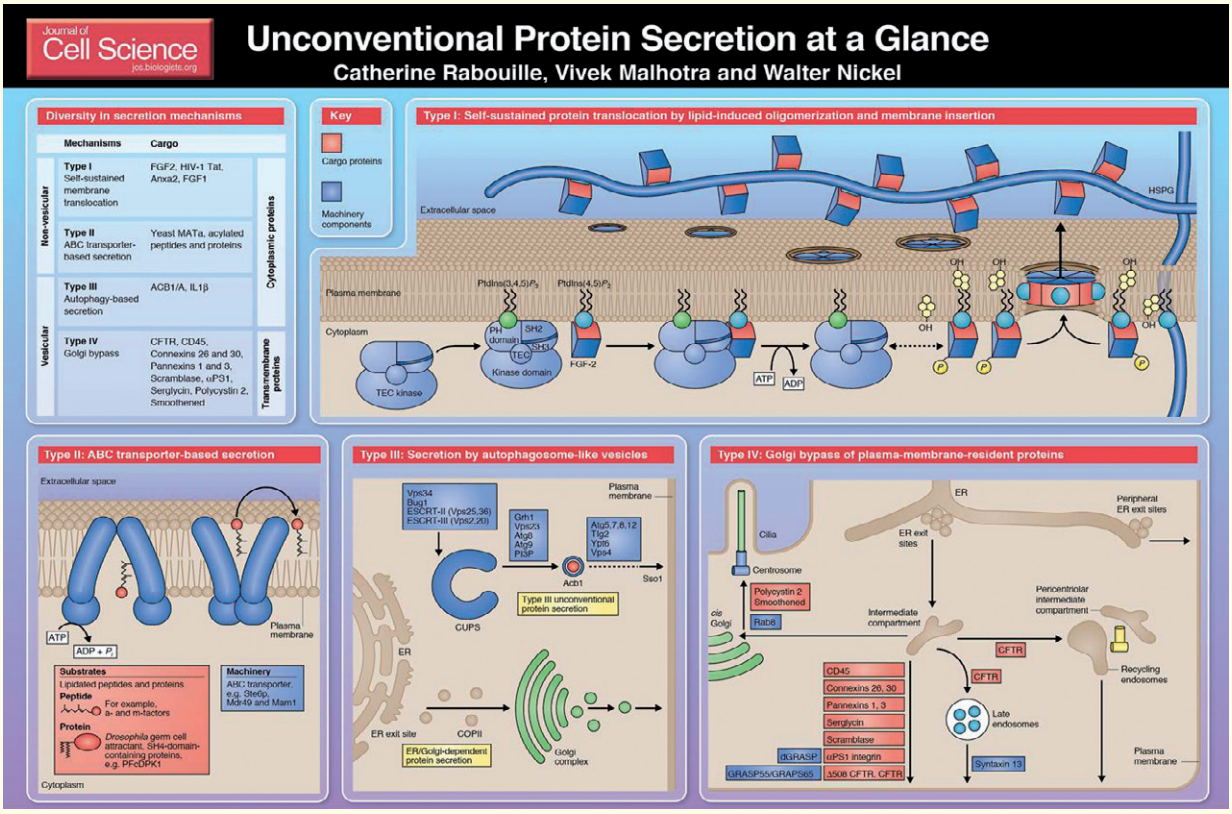

Figure 4.
Poster review article published in the *Journal of Cell Science*. Courtesy of Walter Nickel, UPS Project Leader.

This project aimed at elucidating these 'unconventional pathways of protein secretion' at the molecular level. Advanced techniques such as iTRAQ and SILAC proteomics as well as genome-wide screening using RNA interference were used to identify essential components and reveal the mechanisms regulating unconventional protein secretion.

Scientific highlights

The scientific highlights of this CRP were the:

1) Identification of tyrosine phosphorylation of FGF2 mediated by Tec kinase as a regulatory mechanism of FGF2 secretion (Ebert *et al.*, 2010; joint publication with HD Beer).
2) Discovery of PI(4,5)P_2-triggered FGF2 oligomerization at membrane surfaces that, in combination with tyrosine phosphorylation of FGF2, triggers the formation of a lipidic membrane pore through which FGF2 translocates across the plasma membrane in a heparan sulphate-dependent manner (Steringer *et al.*, 2012).
3) Direct demonstration of the requirement for an intracellular vesicle intermediate in AcbA/Acb1 secretion (Cabral *et al.*, 2010).
4) Identification of the vesicular intermediate of AcbA/Acb1 secretion as autophagosome-like vesicles (Duran *et al.*, 2010)
5) Identification of a large set of yeast gene products that link AcbA/Acb1 secretion to autophagosome-like vesicles, multi-vesicular bodies and the membrane fusion machinery at the plasma membrane (Bruns *et al.*, 2011)
6) Identification of novel substrates of caspase-1 including caspase-4 and demonstration of their involvement in inflammasome activation and regulation of unconventional secretion of interleukin-1β from keratinocytes (Sollberger *et al.*, 2012).

These and other so far unpublished results led to the conclusion that unconventional secretion of FGF2 on the one hand and AcbA/Acb1 and interleukin-1β on the other hand are mediated by distinct and unrelated mechanisms. These cargo proteins therefore define the 2 major types of unconventional protein secretion that are based on direct membrane translocation and secretion through vesicular intermediates, respectively. These structural differences are also reflected at the level of molecular factors required for these processes. For example, secretion of AcbA/Acb1 (Kinseth *et al.*, 2007) and interleukin-1β (HD Beer, unpublished results; see also Dupont *et al.*, *EMBO J,* 2011) depends on GRASP; however, following down-regulation of GRASP55/65 in HeLa cells, FGF2 secretion remains unaffected (Nickel, unpublished results).

Based on the work of AP1 and IP3, a potential link between AcbA/Acb1 secretion and Golgi-independent transport of α-integrin may exist as both pathways depend on GRASP (Kinseth *et al.*, 2007; Schotman *et al.*, 2008). In AP1, the site of action of GRASP in AcbA/Acb1 secretion could be identified as a structure that is not identical but located in close proximity to ER exit sites (Bruns *et al.*, 2011). These findings have been interpreted in terms of a GRASP positive compartment near the ER that may be required for both the formation of autophagosome-like vesicles involved in AcbA/Acb1 secretion and transport of, e.g., α-integrin to cell surfaces (Grieve and Rabouille, 2011; Giuliani *et al.*, 2011). Both AcbA/Acb1 and α-integrin are transported from these sites to the plasma membrane in a Golgi-independent manner and evidence has been reported that these pathways involve endosomal compartments.

The overall results of this CRP have been summarised in a review article putting forward a systematic classification of unconventional secretory processes (Rabouille, Malhotra and Nickel, *J. Cell Sci.*, 2012). Non-vesicular modes were classified as type I (lipid/oligomerization-mediated translocation across the plasma membrane; FGF2; IP1) and type II (ABC transporter-mediated translocation of acylated substrates; not investigated in this CRP). Vesicular modes were classified as type III (secretion by autophagosome-like vesicles; AcbA/Acb1 and interleukin-1β; AP1 and IP2) and type IV (transport of integral membrane proteins to the cell surface in a Golgi-independent manner; IP3).

The mechanisms of other unconventionally secreted proteins are beginning to emerge. For example, unconventional secretion of HIV Tat is likely to be an additional example for a type I mechanism as it has been reported to translocate across the plasma membrane in a $PI(4,5)P_2$-dependent manner (Rayne *et al.*, *EMBO J*, 2010). Therefore, besides the identification of molecular determinants of unconventional secretory processes, this work has provided a framework that will be useful for future studies on various kinds of mechanisms and cargos of unconventional protein secretion.

European added value

This Programme has initiated a number of new collaborations, especially cross-CRP interactions thanks to the EuroMEMBRANE networking activities that would not have happened without the programme. As a number of other national and international activities in the last 5 years, EuroMEMBRANE has clearly contributed to the recognition of lipid biology and membrane research as an important area in life sciences. Therefore, it can be expected that both national and international funding opportunities for membrane research will continue to be available for this important research area in the biosciences.

Overall self-assessment on the accomplishments of the CRP

Each individual project was productive and successful. An important result of this CRP was the recognition of independent pathways of unconventional secretion that differ with regard to both mechanisms and molecular machinery being involved. These results have been summarised in a common review article describing the functional and structural diversity of unconventional secretory processes with a systematic classification that will be useful for future activities in this field (Rabouille *et al.*, *J. Cell Sci.*, 2012).

Selected publications

Rabouille C, Malhotra V, Nickel W. Diversity in Unconventional Protein Secretion. *J. Cell Sci.* 125 (Pt 22): 5251-5255 (2012)

Steringer JP, Bleicken S, Andreas H, Zacherl S, Laussmann M, Temmerman K, Contreras FX, Bharat TA, Lechner J, Müller HM, Briggs JA, Garcia AJ, Nickel W. $PI(4,5)P_2$ Dependent Oligomerization of Fibroblast Growth Factor 2 (FGF2) Triggers the Formation of a Lipidic Membrane Pore Implicated in Unconventional Secretion. *J. Biol. Chem.*, in press

Sollberger G, Strittmatter GE, Kistowska M, French LE, Beer HD. Caspase-4 is required for activation of inflammasomes. *J. Immunol.* 188 (4): 1992-2000 (2012)

Grieve AG, Rabouille C. Golgi bypass: skirting around the heart of classical secretion. *Cold Spring Harb Perspect Biol.* 3 (4) (2011)

Bruns C, McCaffery JM, Curwin AJ, Duran JM, Malhotra V. Biogenesis of a novel compartment for autophagosome-mediated unconventional protein secretion. *J. Cell Biol.* 195 (6): 979-992 (2011)

Giuliani F, Grieve A, Rabouille C. Unconventional secretion: a stress on GRASP. *Curr. Opin. Cell Biol.* 23 (4): 498-504 (2011)

Cabral M, Anjard C, Malhotra V, Loomis WF, Kuspa A. Unconventional secretion of AcbA in Dictyostelium discoideum through a vesicular intermediate. *Eukaryot. Cell.* 9 (7): 1009-1017 (2010)

Ebert A, Laußmann M, Wegehingel S, Kaderali

L, Erfle H, Reichert J, Lechner J, Beer HD, Pepperkok R, Nickel W. Tec kinase mediated phosphorylation of Fibroblast Growth Factor 2 is essential for unconventional secretion. *Traffic* 11: 813-826 (2010)

Nickel W, Rabouille C. Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cell Biol.* 10: 148-155 (2009)

3.5 Lipid-protein Interactions in Membrane Organisation (LIPIDPROD)

Project Leader:

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Funding Organisations:

Austria: *Austrian Science Fund (FWF)*
Czech Republic: *Czech Science Foundation (GAČR)*
Finland: *Academy of Finland (AKA)*
Germany: *German Research Foundation (DFG)*
Switzerland: *Swiss National Science Foundation (SNF)*

Scientific background and objectives

The objectives of this project are:

1) To understand the spatial, nanoscopic organisation of both lipid-anchored and transmembrane proteins within membranes of live cells with special emphasis on proteins that have been claimed to be raft-associated;
2) To understand how these nanoscopic membrane protein assemblies can associate to generate more stable raft platforms with additional functions;
3) To understand how membrane proteins interact with the different lipid species and how lipid-protein interactions contribute to membrane function. One specific issue is to analyse how transmembrane proteins become 'raftophilic';
4) To complement studies in cells by reconstitution studies of selected proteins using simplified model systems such as Giant Unilamelar Vesicles. The goal will be to find the lipid requirements for association with raft domain;
5) To develop a methodology to simulate and model the interplay of lipids and proteins over a multitude of scales in time and space.

Scientific highlights

Together with other CRPs, the Simons group has bridged an extensive arsenal of both experimental and computational simulation techniques to gain insights into the complexity of lipid-protein interactions, glycolipid conformations and membrane partitioning of transmembrane proteins. This holistic approach delivered a number of clear-cut evidences for the bio-functionality of the membrane organisation and specific lipid-protein interactions in modulating and regulating transmembrane proteins. Another highlight was the first demonstration of how glycolipids can be modulated to hide their identity by modulating their head-group conformation. This mechanism brings up a new general principle of glycolipid recognition.

Contrary to current expectations, the Schütz and Stockinger groups observed by the TOCCSL new technology long-lived association of lipid-raft markers with lifetimes of more than a second. The clusters were small, present at extremely high density and diffused rapidly. These new findings indicate that lipid rafts are small fast scanning membrane entities containing a limited selective set of molecules protected by a lipid shell which 1) prevents the interaction of receptors and signalling components in resting cells and 2) guarantees selective assembly and amplification to an efficient signalosome around a receptor upon its ligation. In addition to TOCCSL, both groups developed another technique

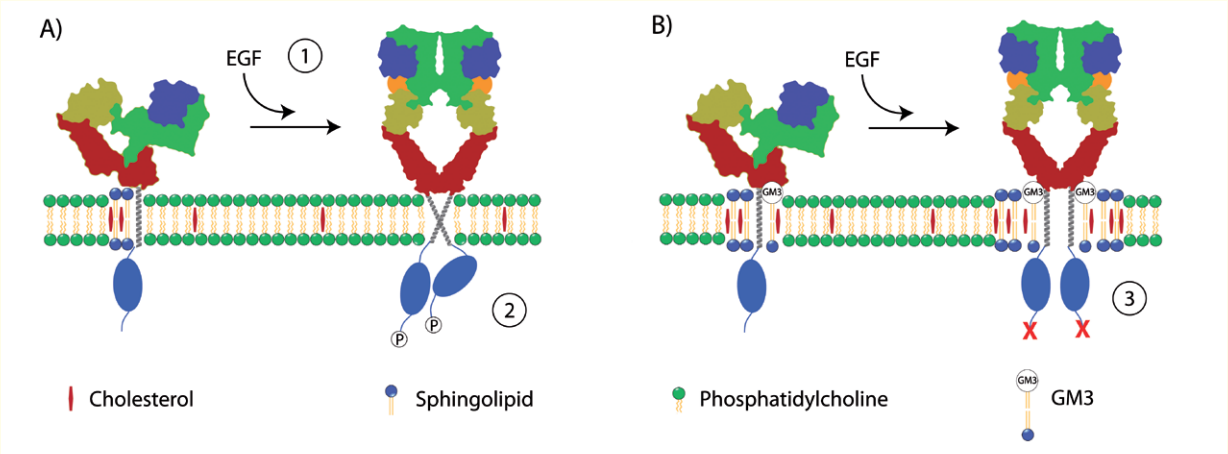

Figure 5.
This scheme demonstrates how A) the EGF receptor is activated to a dimeric autophosphorylated form and B) how the ganglioside GM3 inhibits receptor activation. Courtesy of Kai Simons, LIPIDPROD Project Leader.

based on micropatterning on biochips that allows the quantification of molecular interactions in the plasma membrane of live cells.

Furthermore, the Stockinger group analysed several monomeric green fluorescent protein (mGFP)-tagged palmitoylation-deficient mutants and truncated forms of the Src family kinase Lck, the major kinase in T cell activation. By biochemical and cellular methods, in particular subcellular fractionation, analysis of the phosphorylation status of important T cell receptor signalling components, calcium mobilisation and transcription factor regulation, it was found that palmitoylation of Lck is not only required for membrane anchorage but additionally promotes partitioning into lipid-rafts which in turn facilitates aggregation of the molecule. The latter, particularly Lck dimerisation, is a prerequisite for its functional integrity.

Schwille group's scientific highlights are 1) the development of a sophisticated large-scale modelling that allows the investigation of critical fluctuations in phase-separating membranes, and 2) the influence that membrane-attached proteins and filaments can have on local lipid order, especially actin core structures. Technologically, the group established a free-standing membrane assay with planar, rather than curved, membranes, which allows a better accessibility of high-resolution methods such as AFM and TIRF microscopy to non-supported membranes. This is of particular relevance when studying membrane transformations by proteins.

The van der Goot group in collaboration with the Schütz group tested the hypothesis whether palmitoylation will affect the ability of these proteins to associate with themselves or with partner proteins.

Highlights from the Vattulainen group include the development and validation of simulation models (force fields) for glycolipids and glycoproteins and the use of these models in demonstrating how computer simulations can provide added value for experimental work by showing the mechanisms and physical principles that drive the processes in question. This approach was used to show how cholesterol is able to inhibit the function of certain membrane receptors, how cholesterol gives rise to membrane reorganisation around membrane peptides, and how glycolipids affect membrane receptor assembly.

European added value

EUROCORES programmes such as EuroMEMBRANE are fulfilling an important function in Europe by allowing a bottom-up approach to put together a CRP within a larger network. For membrane research, this approach is unusually productive because of the multidisciplinary nature of the ongoing research in this area. No lab doing cutting-edge membrane research has all the know-hows and technology required to successfully analyse membrane organisation and function. The training aspect of the EUROCORES funding is also very important, thanks to young scientists coming together at the events organised by the CRPs and by the general networking and exchange activities.

During the funding period there has clearly been a change in the appreciation of the lipid-protein interaction field. This is now an upcoming area that is receiving a lot of attention. Kai Simons and Gisou van der Goot organised a Titisee conference on the subject in spring 2012 that attracted a lot of attention. Uenal Coskun and Kai Simons wrote a review for *Structure* (2011) that summarised the state of the field in the following way: "Although cell membranes are packed with proteins mingling with

lipids, remarkably little is known about how proteins interact with lipids to carry out their function. However, novel analytical tools allow comprehending the astounding diversity of lipids in membranes, bringing the lipids back to center stage in membrane research. The potential of specific protein interactions with lipids will be important for unraveling membrane protein structure and function. Progress towards this goal will depend on synergy of different fields that have so far operated without much crosstalk." This CRP has significantly contributed to promoting this crosstalk.

Overall self-assessment on the accomplishments of the CRP

Besides the scientific highlights detailed above, the Horejsi group published biochemical and functional characterisation of several transmembrane adaptor proteins involved on T cell signalling and characterisation their residence in particular types of membrane microdomains.

Simons group studied in detail how the T-cell protein LAT becomes raftophilic, showed that palmitoylation regulates raft partitioning and demonstrated that the transmembrane domain length was important. Raft association was decreased when it was shortened.

The reconstitution studies will further deepen the analysis of lipid-protein interaction required for raft association. Specific lipid requirements for modulating raft protein activity will also be a possible outcome.

The Epidermal Growth Factor Receptor (EGFR) was used as a showcase to find out directly whether gangliosides modulate receptor activity as had been claimed. The Simons group reconstituted the receptor into proteoliposomes of different lipid compositions and demonstrated that the lipid composition had no effect on the equilibrium ligand-binding properties of the EGFR. However, lipids dramatically inhibited kinase domain activation. The effect was very specific and was only seen with the ganglioside GM3, which completely abolished auto-phosphorylation of the receptor. However, the inhibitory effect was only seen in liposomes tuned to phase separate into liquid-ordered (Lo) and –disordered (Ld) domain. These data suggest that GM3 can regulate the allosteric structural transition from an inactive to a signalling EGFR dimer and demonstrate the potential importance of glycosphingolipid-protein interactions, mostly neglected in the cell and structural biology field so far. Altogether, the combined use of new biochemical and biophysical tools will move this field of research to deeper insights into the lipid-protein interaction that form the basis for the dynamics and function of cell membranes.

The simulation studies will hopefully crown the efforts by providing insights into spatial and temporal dynamics of specific lipid-protein couples studied in this CRP.

The Vattulainen group focused on computational studies of biomembrane systems using atomistic and coarse-grained molecular models, with emphasis on atomistic considerations. Three of his projects were carried out in collaboration with the Simons group, and the fourth one together with the Schwille team. These collaborations highlight the significant added value that arises from a close interplay between experimental and computational/theoretical research, which has resulted already by now in 2 major publications, in addition to others that are underway.

In conclusion, the CRP reached most of its goals. The only real deviation concerns the understanding of T cell signalling: this goal is far from being reached but ground work has already been done.

Selected publications

Sezgin E, Kaiser HJ, Baumgart T, Schwille P, Simons K, Levental I. Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat Protoc.* 7 (6): 1042-1051 (2012)

Lingwood D, Binnington B, Rog T, Vattulainen I, Chai W, Feizi T, Grzybek M, Coskun Ű, Lingwood C, Simons K. Cell surface recognition through lipid-dependent receptor display. *Nature Chemical Biology.* 7: 260-262 (2011)

Kaiser HJ, Orlowski A, Rog T, Nyholm TKM, Chai WG, Feizi T, Lingwood D, Vattulainen I, Simons K. Lateral sorting in model membranes by cholesterol-mediated hydrophobic matching. *Proc Natl Acad Sci USA.* 108: 16628-16633 (2011)

Plochberger B, Stockner T, Chiantia S, Brameshuber M, Weghuber J, Hermetter A, Schwille P, Schütz GJ. Cholesterol Slows down the Lateral Mobility of an Oxidized Phospholipid in a Supported Lipid Bilayer. *Langmuir.* 26 (22): 17322-17329 (2010)

Brameshuber M, Weghuber J, Ruprecht V, Gombos I, Horváth I, Vigh L, Eckerstorfer P, Kiss E, Stockinger H, Schütz GJ. Imaging of mobile long-lived nanoplatforms in the live cell plasma membrane. *J. Biol. Chem.* 285: 41765-41771 (2010)

3.6 Molecular Determinants of Sterol-Sphingolipid-Protein Interactions in Living Cells and Organisms (Lipid Specific)

Project Leader:
Professor Elina Ikonen
University of Helsinki, Finland

Principal Investigators:
Dr Suzanne Eaton
Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany
Professor Hans-Joachim Knölker
Technical University Dresden, Germany
Dr Teymuras Kurzchalia
Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
Professor Howard Riezman
University of Geneva – Sciences II, Switzerland
Professor Patrizia Stoppelli
National Research Council, Naples, Italy

Associated Partner:
Professor Daniela Corda
National Research Council, Naples, Italy

Funding Organisations:
Finland: *Academy of Finland (AKA)*
Germany: *German Research Foundation (DFG)*
Italy: *National Research Council (CNR)*
Switzerland: *Swiss National Science Foundation (SNF)*

Scientific background and objectives

Sterols and sphingolipids are major lipids of the plasma membrane and endocytic pathways, found almost exclusively in eukaryotes and differing in their specific structure between species. While the concept of lipid rafts is consistent with a number of findings from model and cell membranes, this hypothesis is not sufficient to explain the biologically observed interdependence between sterol and sphingolipid structures, nor does it help to predict which specific protein-lipid interactions may regulate functions at the cell and tissue level. The molecular determinants governing specific sterol-sphingolipid-protein interactions in cell membranes cannot be predicted from studies in model membranes, where the structural complexity of biomembranes cannot be accurately reconstituted. This project proposes to use instead experimentally amenable model organisms (yeast, flies and worms) as platforms to identify key structural elements and functional consequences of sterol-sphingolipid-protein interactions.

Parallel studies in mammalian cells and tissues will focus on selected aspects of sterol sphingolipid-protein affinities in questions relevant for human physiology and pathology.

A wide combination of state-of-the-art techniques, including genetics, systematic biological phenotyping, chemical synthesis of sterols and sphingolipids, lipid imaging in living cells/tissues and mass spectrometry for lipid analysis will be employed.

Scientific highlights

In a collaborative effort, it was determined which sterols can support growth and development in

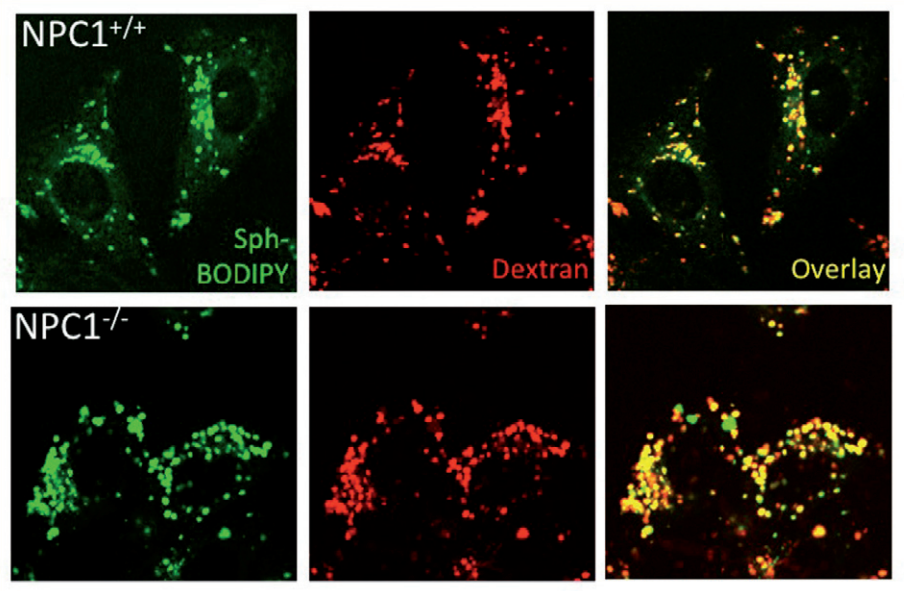

Figure 6.
Fluorescent sphingosine derived from low density lipoproteins (LDL) accumulates in late endosomes independently of NPC1 function.
NPC1+/+ and NPC1-/- CHO cells were incubated with Alexa647-dextran overnight to label the late endosomal compartments, then labeled with 50 µg/mL of Sph-BODIPY/LDL for 1.5 h, chased for 2 h, and imaged live by confocal microscopy. Reference: Blom T *et al. Traffic*, 13: 1234-1243 (2012).
Courtesy of Tomas Blom, University of Helsinki.

Drosophila melanogaster (Carvalho *et al.*, 2010). Sterols can be largely depleted from the flies, but when levels drop drastically they seem to be dispensable for most tissues, except for neuronal cells. Interestingly, sterol depletion leads to an increase in sphingolipids, in particular ceramides. It is possible that sphingolipids replace the structural functions of membrane sterols.

A novel class of glycolipids, the so-called maradolipids, which is specific to the dauer larva in *Caenorhabditis elegans*, was isolated and structurally characterised (Penkov *et al.*, 2010). Follow-up studies indicate that this class of lipids is critical for rendering the larva resistant to severe dessication (Erkut *et al.*, 2011).

Assays were set up for endosomal trafficking, degradation and recycling of fluorescently labeled sphingolipid analogs in mammalian cells (Blom *et al.*, 2010; 2012). These are being employed in ongoing collaborative studies extending beyond the present programme (joint manuscript in preparation). A novel zebrafish model was generated for a human lipid imbalance and a role for lipid droplets was elucidated in regulating unfolded protein response (Hölttä-Vuori *et al.*, *Hum. Mol. Genet.*, 2013).

European added value

The national funding bodies have hopefully increasingly recognised the importance of investigating membrane proteins and lipid-protein interactions. This is expected to translate into funding schemes targeted specifically to investigate this major research interest of the post-genomic era.

A specific example of an international new initiative emerged as a result of the collaboration in this programme: Riezman has employed his genetically engineered collection of yeast strains to produce isotopically enriched sterol species (Shivapurkar *et al.*, 2011). These will, in a future collaboration extending beyond the present programme, be used by the Ikonen group as a basis for generating specific signals for lipids using nonlinear imaging.

EuroMEMBRANE has contributed to increasing the awareness of the high quality of membrane research in Europe and has helped to establish new collaborations outside the EU. As examples, Professor Vladislav Verkhusha from Albert Einstein College of Medicine, NYC, has been recruited by Elina Ikonen *et al.* as a visiting professor to the University of Helsinki in 2012-2016, and Professor Andrew Brown from the University of New South Wales in 2013.

Overall self-assessment on the accomplishments of the CRP

Considering the original, rather ambitious full proposal and the relatively large, geographically widespread and diverse network, this CRP functioned surprisingly well together. Clear synergies and collaborations developed between partners and will be fostered.

The physical proximity of the partners (e.g. Kurzchalia, Eaton and Knölker in Dresden; Stoppelli and Corda in Naples) has helped to form tight collaborations. Yet, also more distantly located groups have, in the framework of the CRP, established collaborations that will extend beyond the present programme (e.g. Ikonen in Helsinki, Riezman in Geneva).

Apart from the most ambitious goal (determination of the structure of a multimembrane span protein with identification of sterol interacting sites), significant advances were made and important objectives reached in all the individual projects.

Selected publications

Carvalho M, Schwudke D, Sampaio JL, Palm W, Riezman I, Dey G, Gupta GD, Mayor S, Riezman H, Shevchenko A, Kurzchalia TV, Eaton S. Survival strategies of a sterol auxotroph. *Development.* 137: 3675-3685 (2010)

Penkov S, Mende F, Zagoriy V, Erkut C, Martin R, Pässler U, Schuhmann K, Schwudke D, Gruner M, Mäntler J, Reichert-Müller T, Shevchenko A, Knölker HJ, Kurzchalia TV. Maradolipids: diacyltrehalose glycolipids specific to dauer larva in *Caenorhabditis elegans*. *Angew Chem Int Ed Engl.* 49: 9430-9435 (2010)

Rajendran L, Knölker HJ, Simons K. Subcellular targeting strategies for drug design and delivery. *Nat. Rev. Drug Disc.* 9: 29-42 (2010)

Pässler U, Gruner M, Penkov S, Kurzchalia TV, Knölker HJ. Synthesis of ten members of the maradolipid family: novel diacyltrehalose glycolipids from *Caenorhabditis elegans*. *Synlett.* 22: 2482-2486 (2011)

Saini R, Boland S, Kataeva O, Schmidt AW, Kurzchalia TV, Knölker HJ. Stereoselective synthesis and hormonal activity of novel dafachronic acids and naturally occurring steroids isolated from corals. *Org. Biomol. Chem.* 10: 4159-4163 (2012)

Hölttä-Vuori M, Salo VT, Ohsaki Y, Suster ML, Ikonen E. Alleviation of seipinopathy-related ER stress by triglyceride storage. *Hum. Mol. Genet.* 22 (6): 1157-1166 (2013)

4.
Networking and Dissemination Activities

Networking and dissemination activities are key characteristics of a EUROCORES Programme. Their aim is to encourage and facilitate scientific collaboration and diffusion across CRPs within a given domain or if appropriate across different domains and programmes.

Networking activities bring together scientists from EUROCORES Programmes and colleagues from other relevant programmes in order to discuss, plan and implement future collaboration and interaction.
Typical examples are:

- Working group meetings, seminars, workshops, symposia, conferences;
- Summer Schools;
- Training programmes and specialised courses;
- Short visits for up to 6 weeks.

Dissemination activities aim at raising awareness on and diffusing results of the EUROCORES Programme.

- Leaflets, posters, publications, books, exhibition booth or stand at a conference;
- Invited sessions at larger conferences;
- Dissemination travel grants to support an active participation at conferences while disseminating the achievements of the Programme.

This section provides an overview of the main EuroMEMBRANE networking and dissemination activities.

2009

- **First Scientific Committee meeting** on 16 June 2009 in Strasbourg, France, organised by the ESF.

This event allowed the first gathering of all Project Leaders, Principal Investigators and (associated) partners / projects members to present the respective aims, methods and approaches of the Individual Projects and CRPs. Thanks to oral presentations from EuroMEMBRANE members and invited internationally renowned researchers, the meeting enabled the discussion of other ongoing (inter) national initiatives in the research field and fostered the networking and collaborative links between the different countries.

2010

- **EuroMEMBRANE launch meeting** on 6-8 April 2010 at Bioquant Center Heidelberg, Germany, organised by Walter Nickel.

The meeting focussed on the scientific topics defined by the 6 CRPs. It gathered a large number of EuroMEMBRANE scientists beyond funded Principal Investigators. All scientific topics and project goals were discussed and cross-CRP activities such as collaborations and workshops were initiated.

- **Scientific Committee meeting** on 7 April 2010, Heidelberg, Germany.

The Project Leaders discussed the EuroMEMBRANE workplan in terms of networking and dissemination activities.

- **Practical Course on "Lipid rafts: Methods for studying membrane organisation"** on 23-31 May 2010 at Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, organised by Kai Simons.
  http://cwp.embo.org/pc10-08/

This EMBO practical course co-sponsored by the ESF combined talks from 12 experienced speakers with lab experiments. 17 young participants could benefit from this hands-on and theoretical course.

- **Workshop on "Fluorescent and lipid probe development"** on 16-20 October 2010 in San Siro, Lago di Como, Italy, organised by Carsten Schultz and Matthias Wymann.

30 participants including young and senior scientists from 3 out of the 6 EuroMEMBRANE CRPs presented their work as lectures or short talks. High-level speakers including Tobias Meyer from Stanford (US), a specialist in phosphoinositide probes, Jin Zhang from Johns Hopkins University in Baltimore (US), best known for genetically encoded fluorescent probes, and Philippe Bastiaens, Director of the Department of Systemic Cell Biology at the Max Planck Institute of Molecular Physiology in Dortmund (DE), were present. Eight collaborations derived from this successful workshop.

2011

- **Summer school on "Biophysics of lipids and lipid-protein interactions: From biomembranes to molecular engineering, drug delivery and imaging"** on 4-11 June 2011 on Utö Island, Finland, organised by Paavo Kinnunen.
  http://becs.aalto.fi/mess/index.html

The summer school was organised in order to expose the students to interdisciplinary approaches in molecular engineering, focusing on the material properties of biomembranes and their constituents, aiming at the development of novel drug delivery systems and sensors. It concretely illustrated how the understanding of the colloidal and biophysical properties of biomaterials allows deciphering the principles of self-assembly in biological systems, organising on different length- and time-scales.

Lectures were delivered by several selected student participants as well as leading experts in the fields, such as Professors Thomas Heimburg (DK), Ole Mouritsen (DK), Stephen White (US), Björn Lindman (SE), Motomu Tanaka (DE) and Erich Sackmann (DE) who is very supportive to the careers of female scientists, in particular from economically and politically strained regions.

Posters had been prepared by 12 out of the 21 students. Awards were given to the best and the most interdisciplinary poster.

- **Workshop on "Probes for membrane systems biology"** on 12-15 October 2011 in San Siro, Lago di Como, Italy, organised by Carsten Schultz and Matthias Wymann.

24 participants presented their work as lectures or short talks. High-level speakers included Takanari Inoue from Johns Hopkins University in Baltimore (US), a specialist in lipid and protein probes, Kazuya Kikuchi from Osaka University (Japan), best known for various fluorescent probes, and Peter Varnai from the Semmelweis University in Budapest (HU), a specialist in cellular lipid detecting techniques. The lively discussions ended in new collaborations, e.g., Carsten Schultz and Matthias Wymann started developing novel fluorescent probes with the Kikuchi lab and Volker Haucke and Kai Simons groups produced excellent results with these membrane-permeant lipid derivatives.

2012

- **Workshop on "High-resolution imaging of membranes and membrane interactions"** on 12-16 March 2012 in Turku, Finland, organised by Pekka Hänninen.
  http://www.bioimaging.fi/euromembrane

The aim of this workshop was to bring together top researchers, post-docs and students in the fields of membrane biology and imaging, and offer them the possibility to learn about the latest developments, especially on super-resolution imaging. The lectures covered imaging applications and latest technical innovations and provided examples of how these techniques are used in the most advanced biological research. The program included 'Meet the Experts' sessions. Over 100 participants registered, including 22 from 5 out of the 6 EuroMEMBRANE CRPs. A joint hands-on training on super-resolution microscopy applications took place on 12-14 March 2012, which allowed 12 PhD students and post-docs test the most modern TIRF equipment along with STED imaging sessions and prepare their own samples.

- **Dissemination Travel Grants to attend the "4th International Singapore Lipid Symposium" (ISLS)** on 13-16 March 2012 in Singapore, organised by Markus Wenk.
  http://www.lipidprofiles.com

The emerging field of lipidomics is driven by technology, most notably mass spectrometry, but also by complementary approaches for the detection and characterisation of lipids and their biosynthetic enzymes in living cells. Leading scientists with various backgrounds in lipid research from all around the world (Eastern Asia, Australia, Europe, US) presented their latest research advances at this symposium that gathered around 100 participants.

Grantees:

Three young scientists:

1) Dr Giovanni D'Angelo (Institute of Protein Biochemistry, National Research Council of Italy, Telethon Italy): "Molecular mechanism of metabolic branching in glycosphingolipid metabolism";
2) Ajay K. Mahalka (Helsinki Biophysics and Biomembrane Group, Department of Biomedical Engineering and Computational Science, Aalto University, Espoo, Finland): "Protein-phospholipid interactions: from biophysics to therapeutics";
3) Adam Orlowski (University of Tampere, Department of Physics, Tampere, Finland): "Role of membrane cholesterol in hydrophobic matching and the resulting redistribution of proteins and lipids".

Experienced researcher:

Dr Harald Köfeler (Medical University of Graz, Austria): "Development of a standardized shorthand lipid nomenclature proposal".

Dr Köfeler presented the proposed shorthand nomenclature for lipids elaborated by the LipidomicNet European-based initiative. It is important to have clear and unambiguous shorthand names beyond the officially accepted systematic naming of lipids in the US Lipids MAPS Structure Database (LMSD). The 4th ISLS started a process of tight collaboration between the European and US lipidomic consortia in terms of standardised lipid nomenclature and other standardisation issues.

- **Conference on "Membrane dynamics in physiology and disease - EuroMEMBRANE International Conference 2012"** on 5-9 June 2012 in Basel, Switzerland, organised by Matthias Wymann.
  http://www.lipidsignaling.org/

More than 210 participants who produced almost 100 posters and 43 talks (plenary, short and flash talks) contributed to a lively and interactive meeting. Highlights were the lectures of the keynote speakers Pietro De Camilli (Yale School of Medicine, US) and Robin Irvine (University of Cambridge, UK). Young researchers showed their potential as well as the impressive progress in the field. Besides participants from EuroMEMBRANE, the audience was very international.

The conference covered various aspects of membrane biology grouped into the sessions "Vesicular Trafficking, Proteins and Lipids", "Lipid Signalling in Health and Disease", "Membrane Organisation and Function", "Lipid Dynamics, Modelling and Visualisation", and "Measuring and Manipulating Lipids". The presented methods and techniques included genetic, biochemical, biophysical, computational, microscopy, chemical and more novel and original approaches.

The conference will have further impact, as a selected number of speakers currently prepare a mini-review series for the *FEBS Journal*.

The conference ended with a discussion about the perspectives of lipid biology.

- **Training Programme and Specialised Course on "Zooming in on plasmamembrane dynamics with Advanced Light Microscopy"** on 11-15 June 2012 in Amsterdam, the Netherlands, organised by Theodorus Gadella.
  http://www.microscopycourse.nl

This 5-day practical advanced course provided 16 students with theoretical background and gave hands-on experience of state-of-the-art membrane microscopy techniques that can be used to elucidate membrane composition, organisation and dynamics. Techniques included confocal and TIRF microscopy, image correlation spectroscopy, fluorescence recovery after photobleaching, Förster resonance energy transfer, fluorescent protein and sensor development and fluorescence lifetime and super-resolution microscopy.

- **Dissemination Travel Grant to attend the FEBS Workshop "Lipids: from lipidomics to diseases and green energy"** on 23-29 August 2012 in Spetses, Greece, organised by Bernd Helms.

Grantee: Maarit Hölttä-Vuori, Institute of Biomedicine, Anatomy, University of Helsinki, Finland (working with Professor Elina Ikonen, Lipid Specific Project Leader).
http://www.febs-lipids.org/Spetses2012/
The topics covered a wide range of lipid research, spanning from cell biology to lipidomics, pathogens, and biofuels. Data were presented both in oral presentations and poster sessions. Time was allocated for "round table discussions". Maarit Hölttä-Vuori made an oral presentation and presented a poster entitled "Alleviation of seipinopathy related ER stress by neutral lipid storage".

- **Dissemination Travel Grant to visit Professor Martin Hof's Fluorescence Group, Jaroslav Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic (ASCR)**, Prague (CZ) on 30 September-25 October 2012.

Grantee: Petteri Parkkila, Institute of Biomedicine, University of Helsinki, Finland.
Supervisor: Professor Paavo Kinnunen, OXPL Project Leader.
Host: Professor Martin Hof, OXPL Principal Investigator.
The visit lasted 18 working days during which Petteri Parkkila, in collaboration with Martin Stefl, Mariana Amaro and Martin Hof, succeeded in constructing a functional system for investigating lateral diffusion in lipid monolayers by fluorescence correlation spectroscopy (FCS). Results show that the FCS-monolayer system provides an effective way to compare how the changes in molecular properties influence model membranes.

- **Short-term visit to Professor Martin Hof's laboratory at the Jaroslav Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic (ASCR)**, Prague (CZ) on 8-23 October 2012.

Grantee: Julia Steringer**,** Heidelberg University Biochemistry Center, Germany.
Supervisor: Professor Walter Nickel, UPS Project Leader.
Host: Professor Martin Hof, OXPL Principal Investigator.
Fibroblast growth factor 2 (FGF2) is a potent mitogen involved in tumor-induced angiogenesis. In order to determine the exact FGF2 oligomeric state at the membrane, antibunching experiments were used. Based on preliminary results obtained during this 16-day visit, a long-term collaboration will be established with Professor Martin Hof, addressing pore size and dynamics by employing z-scan fluorescence correlation spectroscopy (FCS) and a combination of fluorescence-lifetime imaging microscopy (FLIM) and fluorescence resonance energy transfer (FRET) techniques.

- **Workshop on "The future of membrane biology"** on 28 October-1 November 2012 in San Siro, Lago di Como, Italy, organised by Carsten Schultz and Matthias Wymann.

This 3-day workshop follows-up on the previous two organised in 2010 and 2011. It reflects the urgent need in the scientific community to look at the impact of current developments such as novel probes, membrane cell biology, drug development and lipidomics approaches on the future long-term development of membrane biology.

The subjects included lipidomics and analytical methods, lipid signalling, membrane probe development, future of lipid biology, etc. Invited speakers and EuroMEMBRANE graduate students and postdocs were present, with a total of 21 participants. As an example, invited speakers like Philippe Bastiaens (Max Planck Institute, Dortmund, DE) and Tobias Meyer (Stanford, US) are among the most famous scientists in the field of systems biology, while Andrej Shevchenko (Max Planck Institute, Dresden, DE) is one of the few mass spectrometrists worldwide to be able to deliver lipid maps of cells. Speakers presented a short account of their unpublished research before providing their views on where/how their specific area will develop into. All participants were asked to include as many future aspects as possible into their presentations and several discussion rounds addressing future developments were included in the schedule.

5.
Outreach Activities

SYNAPSE

Press releases:
http://www.fmp-berlin.info/fileadmin/Presse_Media/Pressemitteilungen_2011/Pressemitteilung_110805_Small_molecules_engl.pdf
http://www.fmp-berlin.info/fileadmin/Presse_Media/Pressemitteilungen_2011/Pressemitteilung_110801_And_fire-how_nerve_cells_engl.pdf

National / international newspaper articles:
Several press releases and newspaper reports (i.e. The Scientist, The Register (UK), Australia Unlimited, ABC Science, Life Scientist, SiliconInvestor, Bionity), including radio broadcasts and interviews regarding the development of clathrin inhibitors as potential new therapeutics have served to raise public awareness on membrane research and its value for science and society:
http://www.australiaunlimited.com/printpdf/268
http://www.abc.net.au/science/articles/2011/08/05/3285707.htm
http://www.curado.de/Therapieansaetze-Krebs-Molekuele-22611/
http://www.theregister.co.uk/2011/08/05/pitstops_block_viruses/
http://www.bionity.com/de/news/133958/kleine-molekuele-ganz-gross-neue-therapieansaetze-gegen-viren-bakterien-und-krebs.html
http://www.cmri.org.au/Major-discovery-chemicals-that-block-substances-entering-cells/default.aspx

Radio appearance:
http://www.abc.net.au/am/content/2011/s3285897.htm

Patent:
United States Provisional Patent Application No. 61/508,600 (related to von Kleist *et al.*, 2011; clathrin inhibitors)

Website:
http://www.fu-berlin.de/cellbio/eurosynapse

TraPPs

Press releases:
Obtained funding was announced in scientific channels, university news, websites of national funding agencies.

Patents:
Beaufils F, Jacques O, Erhart D, Wymann MP (2010_1), [C16/C40] Rapamycin-based Chemical Entities for Protein Hetero-dimerisation and Membrane Interactions (US Provisional 1004200.0).
Beaufils F, Jacques O, Erhart D, Wymann MP (2010_2), Crosslinkers rendering chemical and biological probes cell permeable (US Provisional 1004200.0).
Beaufils F, Jacques O, Erhart D, Wymann MP (2010_3), Photoactivatable Rapalogues for Protein Hetero-dimerisation, Membrane Interactions, Therapy and Diagnostic applications (US Provisional 1004200.0).
Beaufils F, Jacques O, Erhart D, Wymann MP (2010_4), Phospholipid and carbohydrate

Protein Tag Substrates for Diagnostics and Drug delivery (US Provisional 1004200.0).

Korur S, Beaufils F, Wymann MP (2012_6), Combinations of lysosomotropic or autophagy modulating agents and a GSK-3 inhibitor for treatment of proliferative, inflammatory and degenerative diseases (Europ. Pat. Application, EP12170676).

LIPIDPROD

TV appearances:

Kai Simons. Zukunft mit Zellbiologie gestalten. Südwestdeutscher Rundfunk, teleakademie. 29.04.2012

Petra Schwille, Synthetisches Leben. Scobel 3sat in Mainz. 24.11.2011

Lipid Specific

Launch of the EuroMEMBRANE programme presented at the University of Helsinki as research highlights since 2 EuroMEMBRANE CRP leaders were affiliated with the University, i.e. Elina Ikonen and Paavo Kinnunen:
http://www.helsinki.fi/ajankohtaista/uutisarkisto/7-2009/6-09-26-32

6.
Related ESF Activities

Relevant **ESF Research Conferences** to the field of cell membrane biology took and will take place as partnerships between the ESF and the European Molecular Biology Organization (EMBO) or the Federation of European Biochemical Societies (FEBS). Some were supported by the European Commission as "Human Potential Programme". Relevant conferences are:

1) The **ESF-EMBO symposium** "***Cell Polarity and Membrane Traffic***" (31 March-5 April 2012, Polonia Castle Pultusk, Poland) focussed on cell polarity and vesicle sorting which are fundamental biological processes that impact stem cell function, cancer, bacterial and viral infections, developmental defects and neurological diseases. Early screens in model organisms identified many conserved genes necessary for cell polarisation, but initially these were not recognised as playing any role in vesicular transport. However, interdisciplinary studies from a number of laboratories have revealed exciting and unexpected links between cell polarity establishment and membrane traffic. This conference has been until now the last one of a successful series (the previous one took place in 2009 in Spain).
2) The **ESF-EMBO Symposium** "***Molecular Perspectives On Protein-Protein Interactions***" (25-30 May 2013, Polonia Castle Pultusk, Poland) will bring together scientists from molecular cell biology, biochemistry, structural biology, biophysics and bioinformatics to explore the important field of protein-protein interactions. The ability of proteins to interact fast and with specificity is a basic feature of the complexity of life. The principles governing these interactions are the topic of this conference. This conference is the fourth edition of that successful series (the previous one took place in 2010 in Spain).
3) The conference series entitled "***Biological Surfaces and Interfaces***" has taken place every 2 years since 2005, jointly with EMBO, FEBS and/or the European Commission. The next one, a **FEBS-ESF workshop** (30 June-5 July 2013, Sant Feliu de Guixols, Spain), will cover science and technology of relevance for interfaces between synthetic materials and biological systems or within biological systems – biointerfaces. Rapid progress is driven by a number of growing industrial and clinical applications – biosensors and biochips, tissue engineering and medical implants, stem cell therapies, nanomedicine and drug delivery, medical implants – and by the connected need to understand biointerface and self-assembly processes at a fundamental level. Biologists, chemists, physicists, engineers and physicians will be brought together to exchange ideas.
4) Past individual conferences were also relevant to EuroMEMBRANE such as the ESF-EMBO Symposium "***Emergent Properties of the Cytoskeleton: Molecules to Cells***" (3-8 October 2010, Spain) or the joint EMBO-FEBS-ESF Workshop "***Membrane Dynamics in Endocytosis***" (17-22 September 2005, Spain).

7. Conclusions

In total, 6 out of 11 Review Panel members evaluated the EuroMEMBRANE Programme.

Progress of the CRPs: achievement of CRP goals, integration of teams' outputs

The Programme provided an excellent platform for the individual groups to collaborate. The individual Collaborative Research Projects made good to excellent scientific progress, each of them achieving many of their initial goals. Integration within each CRP also appears to have worked very well, with those teams working best where members had been collaborating before. However, they mostly consisted of close interactions between 2 partners and larger concerted efforts were rarely pursued. Also, there were not as many cross-disciplinary collaborations as would have been possible even within the same CRP (e.g. between cell biologists and physiologists, (patho)physiologists and biophysicists, cell and molecular biologists).

The scientific output from most CRPs was excellent and new advances within the respective membrane biology field have been reported. Among them are new insights into lipid regulation of EGFR signalling (LIPIDPROD), new molecular tools to study PIP signalling (TraPPs), new insights into endocytosis regulation including a first endocytosis inhibitor (SYNAPSE). The work from the SYNAPSE group is well put together with significant discoveries (stonins, pitstops, proteomics) published in high impact journals. The LIPIDPROD group demonstrated that the lipid rafts are of major importance for the spectrum of cell biological to biochemical to biophysical definitions and made this model understandable to the majority of biological scientists, which is a huge step forward in membrane research. The reagents and tools generated by the very active TraPPs goup are highly relevant and used by the project members themselves as well as other groups in the Programme (SYNAPSE, Lipid Specific). The UPS group has documented that the various models of unconventional protein secretion under study may not be related as a single coherent mechanism which is a substantial advance *per se* even if this outcome appears rather disappointing. The remaining groups have demonstrated high productivity with varying degrees of integration but nevertheless greater than expected.

Programme integration of CRPs into the EuroMEMBRANE programme

Interactions between different CRPs increased during the funding period but could have been more intense. For instance, there were few EuroMEMBRANE-derived common publications across CRPs: 1 TraPPs / SYNAPSE and 1 TraPPs / Lipid Specific. This certainly reflects that a 3-year funding period is too short to fully integrate such a large number of investigators in a common effort and to establish effective cross-disciplinary collaborations. The true value of the programme can only be assessed in a couple of years.

All investigators have benefited from EuroMEMBRANE since it would have been difficult for most of them to gather such a large amount of funding from other sources. Without this funding the field would not have advanced and it seems that the Programme had a real stimulating effect on the individual CRPs. In this regard, the most prominent and excellent interaction between SYNAPSE and TraPPs that led to the identification of a new phospholipid involved in the control of endocytosis is certainly the scientific highlight of the

Programme. The impression is however that while most researchers saw this as an obvious opportunity to invest in their own research, they did not fully embrace the chance to establish a pan-European network.

Networking, training and dissemination activities

Interactions within individual CRPs were good but much less developed across the CRPs although all Project Leaders and Principal Investigators from all CRPs participated to all activities.

The main interaction took place at the occasion of 2 Euromembrane meetings, one in Heidelberg in 2010 (kick-off meeting) and one in Basel in 2012 (final conference). There was clearly a high level of networking via meetings, conferences and workshops, particularly in 2012. Due to the development of new technologies in some CRP labs, such workshops are highly useful for training and dissemination of expertise.

TraPPs was seen as outstanding in terms of public outreach and organisation of meetings and events including the final conference in Basel this year.

The environment for young trainees in each of the groups has been exceptional especially considering the cross-disciplinary methods mastered by each group. The most valuable teaching experience for young scientists is to be part of a high quality research network and this "training the next generation" goal was fulfilled. In addition, short visits and dissemination travel grants were offered this year to the least active groups who had less benefited from the Programme networking and dissemination funding.

Potential follow-up activities and future perspectives

As an extension of EuroMEMBRANE is not possible, financial support from the ESF can only come from applications for funding for a Research Conference within the ESF-EMBO partnership (next call in 2013) or for an Exploratory Workshop (next call in March 2013).

A close interaction with the European and US lipidomic consortia (LipidomicNet and Lipid MAPS, resp.) is also encouraged.

It is important to highlight that European funding is key in the field of cell membrane biology and a lack of funding in this research area could be seriously detrimental in the future.

Conclusions and final remarks

The Review Panel took into account (a) the short duration of the Programme (3 years only), (b) the difficulties in getting started due to the unavailability of funding and (c) the fact that funding was limited with many Associated Partners who were not fully supported contrary to what was originally planned. All this was seen as a major hurdle to the full success of EuroMEMBRANE. An extension of funding would have been beneficial for the Programme to achieve its full potential.

Overall the programme resulted in high-quality scientific output and the generation of tools that expanded the scientific knowledge, provided the basis for future investigations, and strengthened the position of European science in this research area. Given the importance of the work from at least some of the groups and the major discoveries indicated, the programme has exceeded expectations.

EuroMEMBRANE did not yet result in a tight European network but this cannot be expected after 3 years of funding. However, the level of participation was high despite a variable activity among Principal Investigators and between CRPs. The true value of the Programme will only become apparent over the next couple of years since some of the recent collaborations look very promising.

Future perspectives

The (still small) lipid membrane biology community is now growing closer together and many tools from the participating laboratories have reached ample distribution to the benefit of more sophisticated experiments, making it possible to unravel this specific research field of biology. Major efforts are necessary to improve analytical methods, ideally for label-free determination of lipids in intact cells. Furthermore, tools are required to manipulate lipid-metabolising enzymes and much more needs to be discovered about the spatial distribution of lipids and the methods used by cells to maintain their inner concentration gradients. The latter subject is still in its infancy. The diversity and complexity of lipid fatty acid composition are totally underestimated and need to be explored in much more detail in order to better understand membrane biology in the future.

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