Featuring cutting edge research projects and enabling technologies of the Netherlands Proteomics Centre
About

The Netherlands Proteomics Centre (NPC) is a strategic collaboration of research groups from seven universities, four academic medical centres and several research institutes and biotech companies. With a scientific programme addressing key areas of proteomics in several projects, and specialised ‘research hotels’, the NPC performs high-quality research and knowledge transfer in an international context. The NPC is part of the Netherlands Genomics Initiative.

In NPC Highlights researchers present progress and results from NPC projects of the scientific programme and the research hotels. NPC Highlights is published by the Netherlands Proteomics Centre.

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Welcome It is my pleasure to present some of the current NPC highlights addressing scientific research, valorisation, education and science communication. This issue nicely demonstrates the broad spectrum of activities endorsed by the NPC.

To start, UK-based artist Charlotte Jarvis has elaborated on the bio-art project ‘Blighted by Kenning’, together with NPC researchers and our communication team, over the last year. This summer we unveiled ‘Blighted by Kenning’ in Suffolk, UK. It was a great success and we are proud to announce that you can now visit the exhibition, which runs until 6 December at Hôtel Droog in Amsterdam. The NPC also participates in the D&A pop-up store, a new concept for public communication coordinated by the Netherlands Genomics Initiative. A small installation of ‘Blighted by Kenning’ will be exhibited at the D&A store as of 15 November 2012. More information about this exciting project can be found on page 24.

Another example of our activities is the strong focus on the training and education of young, talented researchers within the NPC. As in previous years, this summer a couple of PhD students received an NPC grant to visit the EU Summer School in Proteomic Basics in Italy. Furthermore, five NPC researchers visited the BioBusiness Summer School in Amsterdam. You can read about their experiences on page 29.

We are also looking forward to the coming years. On 11&12 February 2013 we will be celebrating the 10th anniversary of the NPC where a great number of internationally renowned speakers will give lectures in addition to other activities. Please visit the NPC website for details of the programme as well as for abstract submission and registration, and mark your calendar to make sure you will be there!

The NPC is now busy preparing a strategy for the future. Part of this is a more integrated approach to biological research by multiple enabling technologies as proposed in the concept of the Dutch Techcentre for Life Sciences (DTL) of which NPC is one of the founding partners. You can find an update of the initiation of DTL on page 30.

We hope you enjoy reading this issue, and please feel free to contact us any time for any questions or suggestions.

Albert Heck, scientific director NPC
The next edition of the NPC PhD day is coming up on 17 January 2013. The NPC is organising a day of lectures, workshops and a lab tour in collaboration with DSM Biotechnology Center in Delft. All NPC affiliated PhD students are invited to attend this day. Attendance is free of charge, but those interested should RSVP by December 3rd. For more information, the programme and registration, please visit the NPC website.

Natural publication
Deletion of genes causes cancer

In a recent Nature paper Hans Clevers’ group of the Hubrecht Institute together with researchers from the UMC Utrecht, Utrecht University and the Netherlands Proteomics Centre describe a gene that limits the growth of intestinal adenomas. The research might yield a lead compound in the treatment of intestinal cancer. Stem cells in the gut continuously provide new tissue. Hans Clevers succeeded earlier in identifying and isolating these stem cells. Together with Madelon Maurice (UMC Utrecht) and Albert Heck (UU, NPC), the Clevers group searched for genes which are only active in the intestinal stem cells. They found RNF43. When this gene is deleted, exponential growth of the intestinal stem cells cause adenomas, a pre stage of intestinal cancer.

Publication: Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors (Nature, 30 augustus 2012, 488, 665-669; doi:10.1038/nature11308)

Visit DTL online

The Dutch Techcentre for Life Sciences (DTL) is a collaborative public-private initiative to provide high-end and pioneer technology to enable ground-breaking research in molecular biosciences.

In essence, DTL is a strongly intertwined network of expert centres among the Dutch universities, university medical hospitals as well as public and private research institutes. NPC is one of these expert centres and founding partner of DTL. To support the development and branding of DTL, the Community and Outreach Team developed a logo and website for DTL (www.dtds.nl).

In addition, a LinkedIn group is opened for discussions. Please join!

Shopping for DNA

The Netherlands Genomics Initiative has launched a new concept: the D&A pop-up store. The store pops up in an empty venue in an arbitrary Dutch city’s shopping street, stays there for a short time, and then disappears again to pop up in another city some days or weeks later. The D&A store travels through the Netherlands until April 2013 and tries to acquaintance as many people as possible with DNA. Visitors can experience DNA, not just as a genetic profile, but as a lifestyle. The NPC participates in this project with a small installation of the bio-art project Blighted by Kenning.

Visit the website of the D&A store to see if the store also pops up in your city!: www.dnastore.nl

NPC Valorisation Voucher
Call for applications

Researchers in proteomics with early stage commercialisation plans are invited to write a proposal for an NPC Valorisation Voucher. The NPC Valorisation Voucher is meant to enable proof-of-concept studies and fill in the gap towards further funding of commercialisation plans (e.g. NGI Pre-Seed).

Projects qualifying for a Valorisation Voucher could address experimental validation, marketing and/or IP related support, required to build a solid business opportunity.

For more information please go to our website or contact Adinda Woelderink, NPC Valorisation Manager woelderink@npc.genomics.nl/+31 30 253 6803.

NPC PhD Day 2013

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The Designers & Artists 4 Genomics Award (DA4GA) highlights and explores the exciting and novel possibilities between design, artistic practice and life sciences. Conceived by the Netherlands Genomics Initiative, CSG Centre for Society and the Life Sciences, Naturalis, and Waag Society, DA4GA aims to stimulate emerging designers and artists to delve into the world of bio-art, and produce new work in close collaboration with the Netherlands most prestigious Life Sciences research institutes, for example in the fields of sustainability, food, health, bio-informatics, agriculture, safety and energy. The second edition of Designers & Artists 4 Genomics is currently showcased in museum Naturalis in Leiden. They are on exhibition until the end of this year.

Second DA4GA exhibition

Dolf Weijers has been appointed full professor with a personal Chair in Biochemistry of Plant Development. The appointment to personal professor is made on the basis of great merit in scientific research and education, as well as remarkable personal qualities. Weijers is a biochemist and developmental biologist and he is involved in NPC’s Research Theme ‘Proteome Biology of Plants’ (T2). Weijers (1976) studied biochemistry at the Hogeschool Enschede and in 2002 received his PhD on the hormonal control of embryo development in plants from the University of Leiden. His studies focus on initial construction of stem cells in plant embryos. For his work on the construction of tissues and stem cells in plants-embryos he received a VIDI grant from NWO (2006) and an ERC Starting Grant (2011). He is also a member of the Young Academy - KNAW.

Dolf Weijers personal professor

In a recent Nature Methods publication NPC scientific director Albert Heck and his colleagues from Utrecht University introduced a new mass spectrometer based on Orbitrap™ technology. This high-sensitive instrument might play a crucial role in the development and use of therapeutic antibodies. In close collaboration with the research group of Alexander Makarov of Thermo Fisher, the inventor of the Orbitrap analyser, the researchers show that protein assemblies of molecular weights over 1 million Da can be analysed with very high analytical resolving power and exquisite sensitivity down to detection of single ions. The new mass spectrometer allows the measurement of a range of important proteins and protein assemblies allowing a detailed analytical footprint of these biologically and medically important molecules. Especially in the fast-growing arena of biopharmaceuticals (e.g. therapeutic antibodies) this new instrument will be important both in research & development and in quality control, to enable such molecularly complex biomolecules to be used safely in the clinic.

Heck: “This new mass spectrometer opens up avenues to measure not only protein-protein interactions, but also covalent and non-covalent binding of small molecules to protein assemblies. Wide-ranging applications may include the direct analysis of drug molecules binding to their targets, and the investigation of post-translational and chemical modifications on intact proteins and protein assemblies.”

Dialogue between science & society

The CSG Centre for Society and the Life Sciences invites all interested to Shaking Science!: the event to meet the life sciences. All on the principal that science meets society and society meets science. During the full month of November 2012 visitors can attend workshops, films, debates, lectures and expositions throughout the country where new technology and society meet each other. Activities that inspire and provide food for thought. The NPC participates in this event with the bio-art project Blighted by Kenning.

Learn more about Shaking Science!: www.shakingscience.nl

Novel mass analyser unravels complex biomolecular structures

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NPC Valorisation Website

Looking for more information on ways to utilise your knowledge? Plans for starting your own company? Looking for (business) events and courses? In search of funding options for early startups? Check out our website! Over the last months the NPC office has been working on the website focused on valorisation. The objective of the website is to give more insight into the NPC valorisation activities and to provide the NPC researchers with information on how to ‘valorise’ their research. The NPC valorisation website is part of the NPC website. www.netherlandsproteomicscentre.nl/npc/valorisation.html
Membrane proteins are involved in many essential cellular processes such as transport of nutrients, sensing of environmental changes, energy transduction and scaffolding of cell structure. Due to their important roles in these processes, they are also linked to various diseases that make them clinically important as potential drug targets. Bert Poolman's research group Membrane Enzymology (part of the Groningen Biomolecular Sciences and Biotechnology Institute (GBB) and the Zernike Institute for Advanced Materials) has accumulated a great deal of experience in the field of membrane translocation processes and membrane proteins in general. Poolman explains: “Our research may generate applications for the drug industry, for example, but our focus is mainly fundamental; we investigate which molecules are involved in transport and try to unravel how they function. Proteomics has helped to determine the focus of our research. For example, if via DNA microarrays we find that the expression of hundreds or thousands of genes in a cell have changed in response to a particular stress, then using proteomics we can find out what this means at the level of proteins and narrow down the number of relevant targets. If you are able to reproduce these changes by engineering of the cell, then you really begin to understand the mechanisms behind the changes.”

Poolman’s team managed to successfully engineer the test organisms *L. lactis*, *E. coli* and *S. cerevisiae*, which have proven to be suitable for hosting the production of membrane proteins.

Many processes in membrane transport still need to be unravelled. “We are not able to change the characteristics of membrane proteins rationally, let alone to adjust the transport mechanisms in a cell,” states Poolman. In his research career he hopes to find that more directed interventions in the functioning of a cell — the field of synthetic biology — are possible. The work complements Poolman’s fascination with unexpected discoveries. “I always tell my students and postdocs to stay alert for deviations in measurements and not to stick to prevailing dogmas. This may lead to unexpected breakthroughs.”
Many research groups have collaborated or are still collaborating with the Poolman group because of its expertise in membrane biology. Being an NPC Research Hotel generated a great deal of collaboration as well. NPC guest researchers can have proteins from highly complex mixtures analysed by mass-spectrometry-based identification, or individual membrane proteins, protein complexes and their modifications can also be characterised biophysically using varying techniques. "Since membrane transport occurs in diverse cellular processes, we have dealt with various topics outside of our own research focus as well."

Super-resolution optical microscopy
To study the dynamics and localisation of processes in cells, Poolman’s group uses super-resolution microscopy and other optical methods, developed with Professor Antoine van Oijen’s Molecular Biophysics group at Groningen. "Using standard optical microscopy techniques, particles smaller than 200-300 nm cannot be discerned. However, with super-resolution one can discern particles down to 10-20 nm, which is in the size range of proteins," explains Poolman. "Thanks to this technique we were able to discover that membrane proteins (coloured with a fluorescence label) are not homogeneously divided over the membrane but reside in clusters. The clustering of proteins may cause different properties. So far, we have been studying ensembles of cells, but our goal is to determine the proteome of a single cell. Using this process one can really determine the differences between individual cells." Recent measurements indicate that seemingly homogeneous bacterial populations are in fact heterogeneous. "We want to know why they are heterogeneous and what causes this heterogeneity. Stochastic processes may play a role; sometimes certain proteins just happen to be present in a lower concentration in one cell than in the other. But occasionally there’s a more sophisticated reason behind it. For example, if some cells are more resistant to high saline conditions than others, their osmoregulatory transporters may be different. If post-translational modifications are involved or molecules are embedded in different lipid environments, two proteins identical in amino acid sequence may each have a different activity. High-resolution optical microscopy on the one hand and high-resolution mass spectrometry on the other will be key to elucidating such differences."

Continuation required
To perform all this research and help other researchers with their membrane protein questions, the Poolman group uses expensive equipment worth more than 1 million euros. "To stay up-to-date in our field, we occasionally need to purchase new equipment, which obviously also costs a lot. Furthermore, to hold on to good proteomics experts we need to offer them steady jobs. Otherwise they very quickly start looking for new jobs. The NPC has helped us enormously in providing the means for this and structuring proteomics research in the Netherlands. However, after 2013 when funding by the Ministry of Economics for NGI and consequently for NPC is discontinued, financial support will be needed to continue research at a high level." Poolman has good hopes that a new initiative like the Dutch Techcentre for Life Sciences (DTL) will be able to provide these means and secure the frontline position of Dutch R&D. Poolman hopes that the name NPC can somehow be maintained, "This has really become a well-known brand name."

Career

Bert Poolman
2008 Program Director Centre for Synthetic Biology
2003 Visiting professor at the California Institute of Technology, Pasadena, U.S.A.
1999 Professor of biochemistry, University of Groningen
1990 Research fellow at the Royal Netherlands Academy of Arts and Sciences (KNAW)
1987 PhD degree (cum laude), University of Groningen

Awards
2011 Elected as Faculty 1000 member
2010 NWO Top-GO subsidy on membrane protein biogenesis
2009 Elected as member of the Royal Netherlands Academy of Arts and Sciences (KNAW)
2008 NGI grant renewal (NPCII) for the Membrane Proteomics Centre
2007 NWO Top subsidy to support the research on ABC transporters and cell volume regulation

Recent key publications

"I always tell my students not to stick to prevailing dogmas. This may lead to unexpected breakthroughs."
How to discover bioactive peptides easier and faster

Martijn Pinkse, Geisa Evaristo, Mervin Pieterse and Peter Verhaert

Unnecessary efforts in structural analysis and bioassay screening are no longer a prerequisite for recognising the most promising peptides from a complex natural sample. Two-dimensional display of liquid chromatography and tandem mass spectrometry runs in combination with specific post-translational chemical modifications can easily identify peptides with potential bioactivity. The method is demonstrated on the basis of the skin secretions of amphibians, which has already led to the discovery of several novel bioactive peptides.

In all of biology, peptides are used as fundamental communication molecules in all branches of the tree of life: from microorganisms all the way to man. Yet it is only in the last few years that the general awareness has steadily been growing of the enormous potential of biologically active peptides, not only as biomarkers for diagnostics but also as (leads for) novel therapeutics. This increased interest is noticeable in today’s applied sciences, with a clear movement within discovery research of both pharmaceutical and (other) industrial biotechnological sciences to explore our planet’s biodiversity for potentially interesting bioactive peptides.

The conventional strategy for novel peptide discovery has been the systematic bioactivity screening of complex natural sources. Bioassays reflecting the activity of interest were performed on chromatographically separated fascinating natural peptide mixtures. Bioactive fractions were consequently purified to homogeneity for primary structure analysis of the respective compounds. Hundreds of biologically active peptides were discovered this ‘classic’ way and, in many cases, multiple peptides with different bioactivities were discovered within the same natural source [1].

Over the past years, the Delft NPC Analytical Hotel has built a unique expertise in peptidomics (the subset of proteomics focusing on the low molecular weight proteins), which in our opinion can play a welcome role in complementing the bioassay based discovery research for novel bioactive peptides.

Focus on bioactivity With its ever increasing sensitivity and specificity, mass spectrometry (MS) became an invaluable analytical tool in peptide biology at the very moment it was introduced into the field. Thanks to MS analyses, it became obvious that the ‘classic’ discovery strategy is seldom all-inclusive, and that many (if not the majority of) peptides from often very rich biological sources remain unstudied. The number of bioassays a certain lab can run is after all limited, so many potentially interesting peptide species are bound to escape the researchers’ attention, which is particularly a pity in cases
What this research is about:

Screening natural sources for pharmaceutical peptides

Short peptides play a crucial role in all sorts of biological processes. They take care of the communication in the body, but can also have an antibiotic, antiviral or fungicidal activity. Therefore peptides have high pharmaceutical potential. “Many peptides have not yet been discovered. They are very often missed by conventional proteomics methods that are not designed to detect them,” says Peter Verhaert, professor of Analytical Biotechnology and Innovative Peptide Biology at Delft University of Technology. “Besides that, the classic study of peptides in natural samples is still a very laborious and time-consuming task. If we were able to recognise peptides that have promising pharmaceutical potential at an earlier stage, it would save a great deal of effort.”

In this article Verhaert and co-workers describe a novel generic method for discovering peptides with bioactive properties in complex mixtures obtained from natural sources which would otherwise remain unrevealed. He uses a combination of liquid chromatography with tandem mass spectrometry analysis and when necessary chemical modifications to identify potentially bioactive peptides. “We look at a set of typical structural elements that are indicators for bioactive compounds. Sometimes we make those visible by giving the sample a chemical treatment and by comparing it with the untreated sample,” explains Verhaert. After identification and characterisation of the promising peptides, final proof of the bioactivity has to be demonstrated using a bioassay.

The method is applicable on all kinds of complex mixtures. Verhaert uses as model system his favourite natural source: the frog. These animals secrete a wide variety of bioactive peptides via glands in their skin as chemical defence. “Their skin is their only method of protection against all sorts of predators and microorganism infections. It is an abundant source of undiscovered peptides with bioactive properties, which include peptides with antimicrobial activity, peptides that inhibit enzymes or paralyse muscles. It also provides peptides for accelerating wound repair, which could in time deliver a more effective drug for wound healing for humans,” Verhaert says.

Figure 1 | Lacking mechanical defence systems, frogs, toads and salamanders are very vulnerable and rely entirely on the activity of the specific collection of peptides (and other biochemical compounds) secreted on their body surface to cope with environmental hazards, including microbial infection and animal predation [picture of Brazilian walking leaf frog, Phyllomedusa burmeisteri, during peptide secretion ‘milking’ by peptidomics research scientist].

where the natural supply of peptides is very precious. It is evident that complex biological peptide collections consist of a mix of the actual bioactive ones as well as their breakdown products and irrelevant protein-derived ‘contaminants’. The brute force structural elucidation of all the (non-tryptic) peptides is something which may become possible in the future, but which today is still unrealistic. Having a system which helps to focus on those peptides that are the most likely to exhibit bioactivity at one’s disposal is therefore extremely useful.

Structural families  Looking a bit deeper into the structural biology of bioactive peptides, one sees that, other than exhibiting a particular bioactivity, they tend to share special characteristics that differentiate them from their inactive counterparts. Indeed, the many bioactive peptides known today can be grouped into several structural families, reflecting that certain structural motifs tend to be common among peptide groups, as well as conserved between various taxa of organisms. These common conserved structural domains frequently coincide with the biologically active site of the peptide under
investment. This is the part of the peptide which fits in the binding pocket of a receptor or within a groove of the catalytic site of an enzyme modifying its activity.

In this respect post-translational modifications (PTMs) are critically important biochemical alterations of a peptide’s primary structure, as these often dramatically affect (part of) their tertiary (3D) structure, making it fit its binder or not.

Using high-end MS based peptide analytics (employing Q-TOF and orbitrap analysers), we developed methods with sufficient MS resolution to specifically screen for these distinctive features.

Frog skin secretions A good example of a rich source of bioactive peptides of sometimes very limited supply is the defensive secretion by the dorsal skin glands of exotic amphibians (see Figure 1). Nowadays, this material — not infrequently hundreds of different peptides per sample — is typically non-invasively collected in the wild. As a third of all amphibian species are threatened with extinction, it is guaranteed that peptide donors remain unharmed and are released back into their habitat immediately after ‘milking’ [2]. Depending on the biological need or ecological niche of a certain amphibian species, bioactivities to be found in its skin secretions are very diverse, and, not seldom, unexpected. Focusing on a limited number of bioactivities, therefore, risks missing many potentially interesting compounds.

2D mapping We have successfully utilised a two dimensional (2D) peptide display, being an image generated from the 2D plot of retention times versus mass-to-charge ratios of LC-MS (peptide) ions, to identify peptides with predefined PTMs/structural features indicative of their potential bioactivity. A conventional one-dimensional LC-MS display (chromatogram with TIC in y-axis) does not give much information on a sample other than a vague indication of its magnitude and complexity. With the publicly available software MSight (www.expasy.org/MSight), LC-MS data are assembled into a more informative two-dimensional image (see Figure 2, for details see [3]) which can be readily interpreted with respect to structural features (i.e. peptide PTMs) of interest to the biologist.

Easily spotted Some PTMs are visible in untreated samples by virtue of the fact that they are not present on one hundred percent of the molecules, which means that for each post-translationally-modified peptide, the unmodified version is also often present in a sample. Depending on the type of PTM, these can be seen at specific mass differences and more or less predictable retention time shifts. A PTM easily spotted this way is C-terminal amidation. Replacement of the free acid carboxyl end (-COOH) of a peptide to its amide (-CONH₂) yields a modified peptide which differs exactly one mass unit from its unmodified variant, whereas its retention only slightly shifts (with the amidated version typically a bit more hydrophobic than the free acid; see insert in Figure 2).

Other PTMs only reveal themselves after specific chemical treatment of the sample. In these cases the differential 2D display comparing treated and untreated samples very elegantly shows peptides with the targeted PTM (see Figure 3). Such treatment can be a very simple one, such as reduction of the sample by dithiothreitol (DTT) or an alternative reducing agent. In this way we have been able to identify and fully structurally characterise novel peptides with both intra- as well as intermolecular disulfide bridges in various frog and toad species [3, 4]. Subsequent bioactivity studies then showed that several of these newly discovered peptides have...
anti-microbial effects [3, 4], whereas others can be classified among protease inhibitors [4].

General conclusion A common tool used to represent LC-MS data as two-dimensional maps can be employed to reveal defined structural features of the MS detected biochemical. Two-dimensional (differential) peptide displays are elegant means for mining complex natural sources of potentially bioactive peptides. They help focus on distinctive structural features of the peptides, thereby becoming part of the discovery strategy of peptides with bioactivity potential.

As such this post-translational modification driven analytical method embodies a complementary ‘reverse’ approach to the bioactivity based discovery of bioactive peptides. One starts by targeting potentially interesting peptides from a natural (microbial or vertebrate) source prior to elucidating their primary structure, and finally analysing their biological activity/pharmacology. We have successfully utilised this strategy to discover novel cysteine-containing peptides in various amphibian skin secretions. This led directly to the elucidation of the full primary structure of several novel peptide bio-molecules that fit into different peptide families [3, 4]. It should be clear that the approach is equally valid for peptide samples of any biological origin.

Being very generic, the method can be expanded — mutatis mutandi — to look for analytes carrying many more PTMs, and we, therefore designated the technique as PTM driven differential peptide display [3]. This is just limited by the creativity of the peptide chemist designing the specific chemical treatment to be performed on the sample between the two LC-MS runs that will be compared.

References
4 Evaristo, G. et al. (2012) The chains of the heterodimeric amphibian skin antimicrobial peptide, distinctin, are encoded by separate messenger RNAs, J. Proteomics: http://dx.doi.org/10.1016/j.prot.2012.09.16

We use liquid chromatography - tandem mass spectrometry (LC-MS/MS) to identify peptides with potential bioactivity in complex mixtures of native peptides from natural sources. As de novo sequencing of non-tryptic peptides is still a very laborious and time-consuming task, it makes sense to recognise the most promising peptides from the mixture first, to avoid spending unnecessary efforts in the energy demanding structural analysis of uninteresting sample components.

The concept is that via a two-dimensional display of a (set of) LC-MS/MS run(s) various interesting features of the peptide sample investigated are elegantly visualised. Against the background of the sample’s complexity, a select set of typical structural characteristics indicative for biological activity are highlighted. Some of these can already be seen directly from the straightforward two-dimensional plot of LC-MS/MS retention time versus mass-to-charge ratio. Others reveal themselves only after selective chemical treatment of the peptide sample, i.e. in a differential two-dimensional display of the LC-MS/MS data of the treated and untreated sample. Employing this strategy, we efficiently localise specific post-translational modifications typical for various classes of bioactive peptides found in nature. Examples include the targeting of carboxyterminally amidated peptides, and (singly or multiply) disulfide bridge-linked peptides in the skin secretion of five different amphibians. This has already led to the discovery and full primary structure elucidation of several novel peptide biomolecules with interesting bioactivities.
Arjen Scholten, Christian Preisinger and Albert Heck

Investigating perturbations induced by kinase inhibitor Gleevec

The effect of Imatinib (Gleevec) on tyrosine kinases in Bcr-Abl positive chronic myeloid leukaemia cells was followed for the first time using a new screening method. The researchers used a combination of stable isotope dimethyl labelling and peptide centric immune-precipitation for targeted quantitative mass spectrometry on tyrosine phosphorylated residues. Their results, as published recently in the journal Leukemia*, reveal new targets in the Bcr-Abl signalling network for intervention.

The human genome encodes for 90 tyrosine kinases and 107 tyrosine phosphatases. Mutation, overexpression, or functional alteration of these enzymes is involved in many diseases including cancer and immunodeficiency diseases. Therefore, targeted analysis of tyrosine phosphorylated residues on proteins and elucidation of their biological role is mandatory for understanding their contribution to signalling networks and, consequently, to pathological processes.

In the last few years, mass spectrometry (MS) has emerged as the supreme tool for studying and characterising phosphorylation events. However, due to the low stoichiometry of protein phosphorylation, methods such as low-pH SCX, TiO$_2$ and IMAC are used for the enrichment of phosphopeptides from complex proteolytic lysates.

Adding yet another layer of difficulty, tyrosine phosphorylation is estimated to represent only about 1-2 percent of all human phosphorylation events with respect to the more frequently occurring phosphorylations on serine (88 percent) or threonine (10 percent) residues. Therefore, these aforementioned approaches are not well suited for the study of tyrosine phosphorylation.

**Immune affinity purification** Several tools have been explored for the specific analysis of tyrosine phosphorylation. Profiling the global tyrosine phosphorylation state of cells can be done using Immobilised Src homology 2 (SH2) domains, which bind selectively to specific tyrosine phosphorylated sites. However, this approach is biased towards those phosphotyrosine proteins that interact with the SH2-containing bait. The development of specific, high affinity antibodies against phosphorylated tyrosines provides an interesting alternative for the global analysis of tyrosine phosphorylation [1]. These antibodies are obtained mostly by immunisation of a suitable organism (e.g. mouse) with phosphotyramine or phosphotyrosine bound to carrier proteins. Traditionally, these antibodies have been used to detect tyrosine phosphorylated residues in a protein of interest by Western blotting. Other applications include enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, cell sorting by flow cytometry and immuno-

* Preisinger, C et al. (2012) Imatinib-dependent tyrosine phosphorylation profiling of Bcr-Abl-positive chronic myeloid leukaemia cells, Leukemia, advance online publication, 14 September 2012; doi: 10.1038/leu.2012.243
Advanced tools for in depth research on leukaemia drug effect

Chronic myeloid leukaemia is characterised by an abnormal increase in immature white blood cells and it is treated with the first line drug Imatinib, also known as Gleevec. The drug suppresses the cell growth and division by inhibiting the responsible kinase (Bcr-Abl) enzyme that is continuously active due to an acquired mutation. “There is a lot of knowledge about the drug and how it works on this kinase in the leukemic cells, but what happens afterwards in the cell is not completely clear,” says Arjen Scholten, assistant professor of the Biomolecular Mass Spectrometry & Proteomics group of Utrecht University. “This kinase enzyme binds specifically with the amino acid tyrosine and attaches a phosphate label to it. It was not easy till now to detect this phosphate modified tyrosine using conventional methods, since only a small percentage of all phosphate modifications present in the cell are of this specific type.”

In this article the authors describe a method that can screen specifically for these modified tyrosine substrates in leukemic cells. “We show that this tool can give insight into a whole protein network at the same time. We identified approximately two hundred modified tyrosine proteins, some already known and many new ones,” Scholten says. He picked one of the newly found proteins to study in more depth in order to demonstrate the potential of this discovery tool.

Since patients develop resistance to Imatinib after a certain period, other targets in the field of the kinase activity could be alternative approaches to circumvent resistance. Scholten explains: “Our studied protein might be an interesting target for a new drug. But that is long term thinking and not really our expertise. We used leukemic cells as a model system to gain new insights into how Imatinib influences related proteins in the leukemic cells. This screening technique can be enormously valuable for understanding biological networks.”

NPC E2: Chemical Approaches to Proteome Biology

Fluorescence. Over the last few years, these antibodies have also been used for the enrichment of tyrosine phosphorylated proteins followed by mass spectrometry analysis [2]. However, detection of the actual phosphotyrosine residue is then hampered by the high presence of unmodified peptides derived from the captured phosphotyrosine modified protein and its co-purifying binding partners.

More recently, the field successfully shifted to the peptide level for the immune affinity purification (IAP) of phosphotyrosine peptides from samples as complex as whole cell lysate digests [3-5]. Most notably, Rikova et al. applied such an approach to investigate tyrosine kinase signalling across 41 lung cancer cell lines and over 150 tumours [5]. The most important difference with the protein level immunoprecipitation is that this approach allows the identification and localisation of the exact phosphotyrosine residue in the protein on every identified protein.

Here we describe how we adopted the IAP method to introduce a robust and simple procedure that allows the identification of hundreds of tyrosine phosphorylated residues within only 6 hours of mass spectrometry analysis time. When coupled to labelling strategies such as iTRAQ, SILAC or dimethylation (used here), this technique is able to extract biologically rich quantitative information on tyrosine phosphorylation driven signalling in differential conditions.

Leukaemia and Gleevec The fusion kinase Bcr-Abl is the major cause and pathogenetic principle of chronic myeloid leukaemia (CML). Bcr-Abl results from a chromosomal translocation that fuses the bcr and the abl genes, thereby generating a constitutively active tyrosine kinase, which stimulates several signalling networks required for proliferation and survival. Bcr-Abl’s oncogenic properties comprise both a kinase and a scaffold protein. A number of Bcr-Abl interaction partners and downstream effectors have been described, improving our understanding of the signalling networks deranged in CML. The ‘core-interactome of Bcr-Abl’ entails seven major interaction partners: GRB2, Shc1, Crk-l, c-Cbl, p85, Sts-1, and SHIP2 [6] (see Figure 2).

The introduction of the Bcr-Abl tyrosine kinase inhibitor (TKI) Imatinib (Gleevec) has been a landmark in the treatment of CML. However, the development of Imatinib resistance poses challenges to the clinical management of CML, and although second generation TKIs can block many Imatinib resistant mutants, they are ineffective against the common T315I mutation. An alternative strategy is to circumvent Imatinib
resistance by targeting downstream pathways essential for transformation. Therefore, it is important to fully understand these pathways. Large-scale (phospho)proteomics experiments have addressed the phosphoproteome of Bcr-Abl positive cells, leading to the identification of an impressive number of serine/threonine-phosphorylated sites (e.g. [7]). Typically, as mentioned above tyrosine-phosphorylation events remained underrepresented in these efforts. Since Bcr-Abl is itself a tyrosine kinase and many of the protein-protein interactions in its network are dependent on tyrosine phosphorylation as well [6], we started out to map these.

Enrichments Therefore we enriched, identified and quantified phosphotyrosine peptides by mass spectrometry [4] in order to examine the effect of Imatinib on the CML blast crisis cell line K562. First, the phosphorylation status and stability of several key proteins involved in Bcr-Abl signalling were evaluated in response to Imatinib (see Figure 1A). We treated cells with Imatinib at concentrations of 0, 1, and 10 µM for 4 hours. Cell lysates were subsequently digested with the proteases Lys-C and trypsin, followed by stable isotope dimethyl labelling of the resulting peptides. The tri-plexed labelling approach allowed us to distinguish three peptide pools (light, intermediate and heavy), which were mixed in equal concentrations. Subsequently, tyrosine phosphorylated peptides were enriched by immunoprecipitation and analysed by LC-MS. Analysis of the quantitative changes in tyrosine phosphorylation yielded 201 unique quantifiable phosphotyrosine peptide triplets belonging to 141 proteins, far exceeding all previous reports [7]. Of these, 87 peptides showed at least a 2-fold down-regulation after treatment with Imatinib (see Figure 1B).

**Down-regulated** Imatinib significantly decreased the tyrosine phosphorylation of many peptides originating from Bcr-Abl and its core interactors (c-Cbl, CrkL and SHIP-2, see Figure 1B and 2A). Furthermore, several proteins that have been shown to play pivotal roles in Bcr-Abl dependent signalling (Gab1, Gab2, Shc1, Crk, ERK-2, STAT5A/B and Yes) displayed reduced tyrosine phosphorylation, often on multiple sites. Also, Src family kinase substrates exhibited reduced tyrosine phosphorylation, e.g. Cortactin, Catenin delta-1, nPKC-delta and Paxillin. Finally, Imatinib reduced the tyrosine phosphorylation of several proteins involved in cytoskeletal regulation, such as MEMO1, Intersectin-2, Catenin delta-1, HEPL, GRF-1, Centaurin delta 2, and Plakophilin, which have not previously been linked to Bcr-Abl signalling.

A motif analysis on the sequences of significantly down-regulated tyrosine phosphorylated sites revealed a distinct enriched motif, YxxP, which is also recognised by Bcr-Abl. In total, 80 percent (23 out of 29) of the peptides harbouring this phosphorylation motif were significantly down-regulated upon Imatinib treatment. The YxxP motif resembles a classic binding site for SH2-domains and a consensus target sequence for phosphorylation by Bcr-Abl [8]. Peptides found in our screen containing this motif include Bcr-Abl (Tyr115 and 128), STAT5A and B (Tyr682 and 699, respectively), several sites in the adapter proteins Gab1 (Tyr259, 373, and 406) and Gab2 (Tyr266, and 409) and all four phosphotyrosine containing peptides of the docking protein HEPL (Tyr174, 195, 244, and 329), indicating that the latter may represent a new Bcr-Abl substrate (see Figure 1B).

Several members of the Bcr-Abl core proteome [6] are adapter/scaffold proteins required for the generation of SH2- or PTB-domain binding sites that lead to tightly regulated protein interactions [9]. Several of these peptides also contain the consensus sequence YxxP of the enriched phosphorylation motif. As these proteins bind to Bcr-Abl, the known and novel enrichment of tyrosine phosphorylated peptides using immobilised phosphotyrosine specific antibodies. The enriched fraction was analysed by LC-MS and changes in tyrosine phosphorylation quantified.

**Figure 1** Exploring the effect on tyrosine phosphorylation upon Imatinib inhibition of K562 cells. A. Overview of the quantitative proteomics workflow. Cells were treated with different doses of Imatinib (0, 1 and 10 µM) for 4 hours, followed by cell lysis and protein digestion. The peptides from each Imatinib treatment were then differentially labelled using stable isotope dimethyl labelling. The three differentially labelled digests were combined, followed by simultaneous enrichment of tyrosine phosphorylated peptides using immobilised phosphotyrosine specific antibodies. The enriched fraction was analysed by LC-MS and changes in tyrosine phosphorylation quantified. B. Quantitative profiles of site-specific tyrosine phosphorylation upon Imatinib inhibition of Bcr-Abl. The changes in tyrosine phosphorylation versus the control are represented on a log scale, marked in red, phosphorylation sites containing the YxxP motif.
Phosphorylation sites detected here may also be putative Bcr-Abl substrates.

**Gab2 interactome** One member of the Bcr-Abl core proteome, the small adaptor protein GRB2, plays a key role in Bcr-Abl signalling through binding to phosphorylated Tyr177 on Bcr-Abl. This is required for activation of the Raf-MEK-ERK pathway in leukemic cells [10]. GRB2 also interacts with the docking proteins Gab1 and Gab2 via GRB2’s SH3 domain and the proline rich regions in both Gab proteins. The observed tyrosine phosphorylations of both Gab1 and Gab2 were found down-regulated by Imatinib. We therefore quantitatively evaluated the changes in the Gab2 interactome in response to Imatinib treatment. Parallel FLAG-immuno-precipitations from K562 cells transfected with either an empty FLAG-vector or a FLAG-Gab2 encoding construct and treated with or without 10 µM Imatinib were performed. This affinity based approach revealed a severe alteration in the Gab2 interactome after Imatinib treatment (see Figure 2A). Whereas GRB2 remained tightly bound, interactions between Gab2 and other components of the Bcr-Abl core-interactome (e.g. Bcr-Abl, SHIP-2, and Shc1) were severely disrupted, suggesting that the Gab2 interactome is dependent on the tyrosine phosphorylation state of this protein. Subsequent immunoprecipitation of endogenous Gab2 and Western blot detection of interactors confirms these results.

The intracellular localisation of Gab2 and the closely related Gab1 protein were evaluated by confocal microscopy. While wild-type GFP-Gab2 smoothly stained the circumference of the cell (see Figure 2B), the phospho-dead mutant where all Imatinib sensitive phosphotyrosine residues were mutated to phenylalanine showed a severe alteration in localisation with speckled staining of the cell periphery in addition to cytosolic staining. Thus, tyrosine phosphorylation of Gab1 and Gab2 and the subsequent ability to promote the formation of a complex with Bcr-Abl and other core members appears to be required for appropriate membrane localisation. These observations strongly advocate an involvement of Gab2 in the assembly of the Bcr-Abl signalling network at the cell periphery and the ability of the core proteome to transform cells.

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**summary**

To probe the effect of Imatinib on tyrosine kinase Bcr-Abl positive cells, we applied a targeted quantitative mass spectrometry based proteomics approach. We used phosphotyrosine specific immuno-capturing to systematically screen for Imatinib sensitive tyrosine phosphorylation events in K562 cells. Our data reveal that several core Bcr-Abl interactors are putative direct substrates, but also new putative targets are discovered. In following up a novel Imatinib inhibited target, the adaptor protein Gab2 was found to regulate localisation of the Bcr-Abl core complex to the cell periphery in a phosphorylation dependent manner, enabling us to add crucial new dimensions of detail to the Bcr-Abl signalling network. This work provides insights into how protein phosphorylation influences protein networks and shows which widespread effects a kinase inhibitor may have on the network biology of this leukemic cell.
Serena Di Palma and Shabaz Mohammed

Accelerating towards ultrasensitive proteome analysis

One of the difficulties with current proteomics analysis methods is the need for samples of hundreds of thousands or millions of cells. In many cases, this amount of cells is not available, for example analysing adult stem cells, tumour biopsy or samples obtained by laser microdissection. A new 2D approach using hydrophilic separation as the first step gives excellent resolution and allows for more sensitive proteome analysis.

In the last decade, the field of proteomics has rapidly progressed and elicited considerable interest. Continuous technological advances in mass spectrometry (MS) instrumentation, peptide fractionation strategies and bioinformatics have led to increasing knowledge of proteomes. However, improvements are still needed, especially for the investigation of limited amounts of material such as laser microdissected tissues or specific subsets of cells obtained through flow cytometry [1]. A suite of special analytical tools is required to deal with under-represented (or limited) samples and to further the single-cell single-proteome dream.

We believe that a new peptide separation strategy based on hydrophilic interaction liquid chromatography (HILIC) will help to accelerate proteomics towards a high level of depth in regard to sensitivity, proteome coverage and comprehensivity. Here we highlight how HILIC can be implemented into shotgun proteomics, to find applications in situations where limited sample availability represents a challenge.

Advantages of HILIC HILIC is a relatively new player in the proteomics field, providing particular advantages in comparison to more established techniques such as reversed-phase (RP) and ion-exchange (IEX) chromatography [2]. First, the high organic composition of the buffer, with low salt concentration or volatile salts, makes HILIC especially suitable for electrospray ionisation, potentially increasing its sensitivity. Second, the special affinity for polar compounds allows the analysis of highly hydrophilic species that would otherwise be lost under RP analysis. Third, the high orthogonality or complementarity to RP makes HILIC an ideal candidate in multidimensional approaches for the analysis of complex peptide mixtures, representing an excellent alternative to strong-cation exchange (SCX) as a first dimension [3]. All these reasons have contributed to an exponential increase in the application of HILIC chromatography in proteomics, especially for the analysis of polar protein post translation modifications (PTMs) such as glycosylation and phosphorylation [4].
Analysing just a single cell among thousands of others is the holy grail of proteomics nowadays. It would certainly help in diagnosing cancer, for example. The study of cell-to-cell communication would also greatly benefit from such an approach. Current proteomics research requires hundreds of thousands or even millions of cells per sample. Common methods for peptides analysis using mass spectrometry need these amounts to accurately identify large numbers of peptides.

Serena Di Palma spent her PhD study finding new peptide separation strategies and, more importantly, miniaturising those systems, with the aim of developing analytical methods that use small sample sizes. She replaced the commonly used chromatographic separation method known as SCX (strong-cation exchange) with a different chromatographic method only recently introduced in proteomics called HILIC (hydrophilic interaction liquid chromatography). HILIC separates peptides mainly by hydrophilicity and to a lesser extent by charge. “This means that peptides do not elute in big clumps (clusters of similar charge) but more separated,” says Di Palma. Separating hydrophilic peptides has become more important in the last years because the interest of proteomics has shifted towards analysis of post-translational modifications of proteins. These are usually polar modifications such as glycosylation and phosphorylation, generating samples with a large amount of hydrophilic peptides.

Di Palma designed a miniaturised set-up using a zwitterionic HILIC, as the peptide fractionation step, combined with reversed-phase (RP) chromatography in a 2D system. “Having zwitterionic stationary phases means that the total charge of the material will remain zero, which gives a special advantage for analyses performed at different pH values,” says Di Palma. She compared her results to a traditional proteomics analysis and found that many more proteins could be identified using the HILIC separation step before applying the RP chromatography. The protein identification matched results from large scale proteomics analysis. The researchers think that this method can be very useful for analysing small samples obtained by laser microdissection or flow cytometry.
causes sample loss and contamination. We showed that this 2D approach combines excellent resolution in both the first and second dimension. Furthermore, it allows for more sensitive proteome analysis in comparison to current methods, such as SCX, that require more input material. In fact, when we analysed prototypical cancer cell lines (HeLa) employing a mere few micrograms of starting material, we could achieve proteome coverage comparable to 'large scale' strategies.

Application to stem cells The identification and characterisation of downstream Wnt target genes led to the discovery of Lgr5, a gene that is uniquely expressed in the stem cells of several adult tissues such as intestine, hair follicles and stomach. A breakthrough in the study of this gene has been the generation of a mouse strain in which GFP has been knocked into the Lgr5 locus. Moreover, a cell sorting method based on FACS has been established that allows a high enrichment (>95%) of these GFP-marked adult colon stem cells, albeit in rather small quantities for proteomics studies. To achieve a targeted proteomics analysis at an ultrasensitive level, optimised methods are required. These include a combination of specific sample preparation for maximal recovery with sensitive methods for separation, detection and identification of all possible peptides/proteins. Thus, we probed the sensitivity of our new 2D technology with a real small-scale sample scenario, and analysed FACS-obtained colon stem cells, directly after tissue extraction [6]. A schematic workflow is summarised in Figure 2.

A population of 30,000 colon stem cells was FACS sorted and subsequently digested using Lys-C and Trypsin. We started with a total of 30,000 cells to have sufficient material for a systematic evaluation using one-dimensional (1D) and two-dimensional (2D) separations. A portion of the sample corresponding to 5,000 cells was subjected to a standard 1D RP nanoLC-MS analysis using a rather long five hour gradient elution. This allowed the identification of 380 proteins and 759 unique peptides, in line with our expectation. Afterwards, we applied our 2D HILIC-RP strategy to the analysis of 10,000 colon stem cells and compared it with the 1D RP-MS analysis. The HILIC fractionation led to 27 fractions, wherein half of each fraction was further analysed by RP-MS. Effectively, considering the overall workflow, we analysed the same amount for both 1D and 2D approaches, consisting of 5,000 stem cells. Combining all the data from the 2D strategy allowed the cumulative identification of 15,775 unique peptides, originating from 3,775 proteins (see Figure 3). Within each fraction, we reported the number of unique peptides and also evaluated the distribution of peptide charge (2+, 3+, 4+ and 5+), as indicated in Figure 3a. Although 2+, 3+ and 4+ charged peptides eluted over quite a number of fractions, a trend of an increase in peptide charge in the later fractions was observed.

A valid alternative to current methods Comparing this result with the 1D analysis, we reached a 20-fold and 10-fold improvement in peptide and protein identifications, respectively. The proteome coverage we achieved at the time rivalled that reached by current state-of-the-art proteome analyses that often require several millions of cells. A detailed comparison between the two strategies revealed that only five (out of 380) proteins present in the 1D analysis were not observed in the two-dimensional ZIC-cHILIC-RP experiment, illustrating that very little sample loss occurs incorporating the additional

Figure 2 | Schematic representation of the employed workflow for the isolation of adult colon stem cells from mouse intestine and the subsequent proteomics analysis performed by the 2D HILIC-RP or 1D RP strategy.

Figure 3 | Analysis of FACS-sorted colon stem cells with a 2D ZIC-cHILIC approach and a 1D RP strategy. (A) The cumulative number of identified unique peptides and proteins and distribution of unique peptides (including peptide change) across the HILIC fractions and the 1D RP run. Venn diagrams showing the overlap of identified (B) unique peptides and (C) protein between the 2D (reported as combined fractions) and 1D approach.
separation step (see Figure 3c). In addition, only 101 peptides (corresponding to approximately 0.6% of the total of 15,775 peptides) found in the 1D analysis were not identified in the 2D experiment, which potentially may be attributed to the incompatibility of certain peptides with the HILIC solvents. In general, both Venn diagrams (see Figure 3b and 3c) showed large overlaps, suggesting that the peptides identified in the 1D RP analysis are merely higher abundant peptides that are all also detected in the ZIC-cHILIC-RP strategy. A further comparison between the obtained proteome data and previous microarray experiments from the same colon stem cells confirmed the quality of our method, showing that 95% of the proteins detected in this study were also found to be expressed at the mRNA level. Furthermore, 21% of the GFP+ colon stem cell specific genes could be detected in our proteomics screen, and two of the identified proteins, CD44 and EphB3, have been reported to be expressed highest at the bottom of the crypt, where the colon stem cells reside. A Gene Ontology analysis for molecular function and biological processes of the 3,775 detected proteins revealed that the 2D strategy covered all the most important functional categories, including, for instance, proteins involved in transcription mechanisms, in receptor activity and in translation regulation activity.

Major step forward Notwithstanding the huge advances made in proteomics in the last decade, sensitive technologies are still needed to access proteome-wide data for a limited number of cells, for instance originating from laser microdissections, flow cytometry, tumour biopsy and other scenarios where sample material will be a limiting factor. We demonstrated that the 2D HILIC based strategy developed in our laboratory leads to a significant reduction of sample complexity with nearly negligible sample loss. The approach described here represents a major step forward towards a more sensitive proteome analysis. We propose to introduce ZIC-cHILIC as a fractionation method in shotgun proteomics strategy for the analysis of only a few thousands of cells. [7] Furthermore, this strategy, in combination with metabolic or chemical labelling based quantification [8], can investigate and compare with high-sensitivity specific cell proteomes.

References

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summary

In proteomics, multidimensional liquid chromatography combined with mass spectrometry has become a standard technique to reduce sample complexity and tackle the vast dynamic range. Such fractionation is necessary to obtain a comprehensive analysis of biological samples such as tissues and cell lines. However, extensive fractionation comes at the expense of sample loss, hampering the analysis of limited material. Here we describe a highly sensitive two-dimensional chromatographic strategy based on a combination of hydrophilic interaction liquid chromatography (with a zwitterionic stationary phase) and reversed-phase chromatography. This strategy allows shotgun proteomics analysis with minimal sample loss. We applied this technology to the analysis of a limited number of FACS-sorted colon stem cells extracted from mouse intestine, obtaining proteome coverage comparable to current methods that generally require 100-fold more starting material. We propose that this alternative multidimensional chromatographic technology will find ample application in biomedical and biological research, such as in the analysis of distinct cellular populations obtained by laser microdissection or flow cytometry.
Genotoxic stress affects protein synthesis through regulation of eEF2K

Eukaryotic elongation factor 2 kinase (eEF2K) is a crucial enzyme in repressing translation elongation. We have recently discovered this protein as a substrate of the SCF$^\text{TrCP}$ ubiquitin ligase using a combined mass spectrometry-based protein interaction screening with an in-house developed technique for phosphopeptide analysis. Focusing on the biological functions of eEF2K, we investigated how the enzyme is controlled by genotoxic stress and how this leads to a decrease in translation elongation and protein synthesis.

Cells activate surveillance molecular networks known as DNA damage checkpoints to protect their genome from environmental and metabolic genotoxic stress. Depending on the type and extent of DNA lesions and the cellular context, damaged cells with an activated checkpoint can undergo senescence, die by apoptotic cell death, or repair the damaged genome and, after checkpoint termination, resume their physiological functions. Genotoxic stress has a greater effect on gene expression at the level of mRNA translation than at the level of transcription, probably because protein synthesis requires about 40% of the total cellular energy, and cells need to couple stress responses to metabolic demands. Therefore, in response to genotoxic stress, cells aim to preserve energy by reducing protein synthesis in order to be able to repair the damage. Despite the major impact of genotoxic stress on mRNA translation, no information is available on how translation elongation is affected by genotoxic stress, and there have been few studies directed toward understanding the regulation of protein synthesis by the DNA damage response.

Translation elongation Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that integrates growth and stress signals and promotes protein synthesis. The mTOR pathway promotes translation elongation by inhibiting eukaryotic elongation factor 2 kinase (eEF2K), which phosphorylates and inactivates eEF2, a factor that mediates the translocation step of peptide-chain elongation. The activity of eEF2K is controlled under various conditions. For example, stimuli that induce protein synthesis trigger the inactivation of eEF2K and the subsequent dephosphorylation of eEF2. In contrast, deficiencies in nutrients or energy lead to activation of eEF2K and impairment of translation elongation.

In addition to this level of regulation, eEF2K abundance is controlled by the ubiquitin-proteasome system. In this system substrates are tagged by covalent attachment of multiple ubiquitin molecules and then the polyubiquitylated proteins are degraded by the 26S proteasome. Several enzymes are involved in this system, but the E3 ubiquitin ligases represent the essential regulators of ubiquitylation because they physically interact with target substrates. Although it is known that
Subsequently, by reciprocal immunoprecipitation with FLAG-HA-eEF2K, we recovered peptides corresponding to βTrCP1 and βTrCP2. This is not surprising since βTrCP1 and βTrCP2 share identical biochemical properties and substrates. The WD40 β-propeller structure of βTrCP binds its substrates (like eEF2K) through a diphosphorylated motif (phosphodegron) with the consensus DpSGXX(X)pS. The generation of several eEF2K mutants with replacements of residues within this motif showed that the motif surrounding Ser441 and Ser445 is highly conserved and required for the binding of eEF2K to βTrCP (see Figure 2A and 2B). Furthermore, we also showed that phosphorylation is required for the interaction of eEF2K with βTrCP. An eEF2K peptide containing phosphorylated Ser441 and Ser445 in-

**What this research is about:**

**How the ubiquitin-proteasome system controls fundamental cellular functions**

"Daniele Guardavaccaro started as a new group leader at the Hubrecht Institute in 2008 and came to us with a very interesting biological question," says Teck Yew Low, researcher at the NPC and Biomolecular Mass Spectrometry and Proteomics group (Utrecht University) and highly experienced in new techniques for analysing proteins.

"Guardavaccaro wanted to know which proteins bind to specific enzymes, known as E3 ubiquitin ligases. " These enzymes tag proteins that need to be destroyed by attaching a small protein called ubiquitin to them. Next, a complex called proteasome seeks out and destroys all molecules with the ubiquitin tag. By finding proteins bound to E3, one may discover new targets that are marked for destruction by the ubiquitin-proteasome system.

Guardavaccaro, Low and colleagues identified many novel E3 binding proteins. One of them, an enzyme known as eEF2K, is a key repressor of mRNA translation — the process by which mRNAs are translated into proteins. The researchers zoomed in on eEF2K and found important details on how mRNA translation is regulated. Translation is a process that requires a considerable amount of energy. "We found that when the cellular DNA is damaged, for example upon exposure to chemical mutagens, the repressor of mRNA translation (eEF2K) is rapidly activated and translation is temporarily slowed down. As a consequence, a large amount of energy is saved and used by the cell to fix the damaged DNA. Once the damaged DNA is repaired, the repressor of translation is first tagged by ubiquitin and then destroyed by the proteasome. As a result, mRNA translation is resumed," explains Low.

In this research, Low and colleagues also carried out extensive analysis of proteins modified by phosphate groups (phosphoproteins). "Regulation of proteins by phosphorylation is the prevalent mode of regulation of protein function and its study is essential in our research. We frequently apply a number of techniques recently developed in our lab to enrich for phosphoproteins and facilitate their analysis by mass spectrometry." Low and Guardavaccaro are currently continuing their collaboration to analyse other E3 binding proteins.

**NPC T1: Cancer Proteomics**

![Figure 1](image-url) The structure of the SCFβTrCP ubiquitin ligase and its phosphorylation-dependent interaction with the substrate. The F-box protein βTrCP is the substrate receptor subunit that recruits specific substrate proteins. Through its WD40 β-propeller structure, βTrCP recognises a diphosphorylated motif with the consensus DpSGXX(X)pS in which the serine residues are phosphorylated to allow interaction with βTrCP.

eEF2K abundance is controlled by the ubiquitin-proteasome system [1], the biological importance of the ubiquitylation and degradation of eEF2K and the identity of the E3 ubiquitin ligase involved are unknown.

**Proteomics screening** To identify substrates of the ubiquitin ligase SCFβTrCP (see Figure 1), we used immuno-affinity chromatography followed by tandem mass spectrometry (MS/MS) [2]. First, we expressed FLAG-hemagglutinin (HA) epitope-tagged βTrCP2 (FLAG-HA-βTrCP2) in human embryonic kidney HEK293T cells and analysed by MS proteins that co-purified with FLAG-HA-βTrCP2 after sequential FLAG and HA immunoprecipitations. We identified nine unique peptides derived from eEF2K [3,4]. Subsequently, by reciprocal immunoprecipitation with FLAG-HA-eEF2K, we recovered peptides corresponding to βTrCP1 and βTrCP2. This is not surprising since βTrCP1 and βTrCP2 share identical biochemical properties and substrates. The WD40 β-propeller structure of βTrCP binds its substrates (like eEF2K) through a diphosphorylated motif (phosphodegron) with the consensus DpSGXX(X)pS. The generation of several eEF2K mutants with replacements of residues within this motif showed that the motif surrounding Ser441 and Ser445 is highly conserved and required for the binding of eEF2K to βTrCP (see Figure 2A and 2B). Furthermore, we also showed that phosphorylation is required for the interaction of eEF2K with βTrCP. An eEF2K peptide containing phosphorylated Ser441 and Ser445 in-
teracted with β-TrCP1, but the corresponding nonphosphorylated peptide was unable to do so. To analyse the phosphorylation of the degron in vivo, we used an in-house developed technique based on Ti(4+)-immobilised metal ion affinity chromatography (Ti-IMAC) as described [5] in combination with orbitrap mass spectrometry.

To test whether SCFβ-TrCP was directly responsible for the polyubiquitylation of eEF2K, we reconstituted the ubiquitylation of eEF2K in vitro. Immunopurified β-TrCP1, but not an inactive β-TrCP1 mutant, induced the in vitro ubiquitylation of eEF2K.

Crucial inhibitor Since eEF2K is a crucial inhibitor of translation elongation and because inhibition of protein synthesis is a common response to stress conditions, we examined the abundance of eEF2K in response to genotoxic stress. U2OS cells were synchronised in the G2 phase of the cell cycle and then treated with a low dose of doxorubicin to activate the G2 DNA damage checkpoint. Subsequently, cells were allowed to recover from the DNA damage checkpoint after extensive washing in a drug-free medium. The abundance of eEF2K steadily decreased during checkpoint silencing. A similar pattern of changes in eEF2K abundance was observed in experiments in which ionising radiation was used. Moreover, eEF2K degradation was inhibited when β-TrCP expression was silenced in U2OS cells during checkpoint silencing. Using a phosphospecific antibody that recognises eEF2K only when it is phosphorylated on Ser441 and Ser445, we were able to show that phosphorylation of the eEF2K degron is induced by DNA damage in G2 and preceded degradation of eEF2K. Together, these findings imply that β-TrCP targets eEF2K for degradation upon checkpoint silencing. The kinase eEF2K controls translation elongation by phosphorylating eEF2 on Thr56, thereby inactivating it. The affinity of eEF2 for the ribosome is reduced when Thr56 is phosphorylated and therefore the ribosome–eEF2 complex is not formed. Our results indicate that phosphorylation of eEF2 on Thr56 increases upon checkpoint activation and decreases during checkpoint silencing. Increased phosphorylation of eEF2 on Thr56 in response to DNA damage is mediated by eEF2K. This was confirmed by our experiment in which eEF2K expression was silenced in U2OS cells. The cells were synchronised in G2 and then pulsed with doxorubicin to induce DNA damage; eEF2K knockdown blocked the DNA damage-induced phosphorylation of eEF2 on Thr56. The kinase eEF2K is phosphorylated on Ser398 by AMPK (adenosine monophosphate-activated protein kinase), a phosphorylation event that leads to eEF2K activation. AMPK is activated by the products of two p53 target genes, Sestrin1 and Sestrin2, in response to genotoxic stress. Using an antibody that specifically recognises eEF2K phosphorylated on Ser398, we showed that in response to genotoxic stress, Ser398 was phosphorylated. Inhibition of AMPK prevented the increase in eEF2K phosphorylation on Ser398 and eEF2 phosphorylation on Thr56 in response to genotoxic stress. Together, these data indicate that genotoxic stress in G2 cells stimulates AMPK, which phosphorylates eEF2K on Ser398, causing its activation. In turn, activated eEF2K phosphorylates eEF2 on Thr56, blocking its activity.

Figure 2 | A. The β-TrCP-eEF2K interaction depends on a conserved phosphodegron. Ser441 and Ser445 in eEF2K are required for the association with β-TrCP1. Top: schematic representation of four putative eEF2K phosphodegrons. Bottom: HEK293T cells were transfected with FLAG-tagged β-TrCP1 and with either an empty vector, HA-tagged eEF2K wild type (WT), or mutants, treated with MG132, and lysed. Whole-cell extracts were subjected to either immunoblotting or immunoprecipitation with anti-FLAG resin and subsequent immunoblotting. The eEF2K(S441A/S445A) mutant immunoprecipitated less with β-TrCP1 compared to wild-type eEF2K and the other mutants. B. Alignment of the amino acid regions corresponding to the β-TrCP-binding motif (highlighted in yellow) in previously reported β-TrCP substrates and eEF2K orthologs (top). Amino acid sequence of the eEF2K double mutant is shown (bottom).

Figure 3 | Model of eEF2K regulation in response to genotoxic stress. Blue lines represent activated proteins, and red indicates inactive or degraded proteins.
Resuming protein synthesis  Since eEF2K has autophosphorylation activity [6], we hypothesised that eEF2K, once activated, may autophosphorylate its degron, thus triggering the binding of eEF2K to βTrCP. Indeed, immunopurified wild type eEF2K could autophosphorylate on Ser441 and Ser445 and bind to βTrCP in vitro whereas a kinase dead eEF2K mutant could not. We then wanted to know whether βTrCP-mediated degradation of eEF2K is required to resume protein synthesis upon checkpoint silencing. Cells expressing the degradation-resistant form of eEF2K that were exposed to genotoxic stress displayed a slower decrease in phosphorylation of eEF2 on Thr56 during silencing of the DNA damage checkpoint. These cells also displayed a decreased rate of global protein synthesis during checkpoint silencing when compared to control cells.

Initiation and silencing  In this article we show that upon activation of the DNA damage checkpoint, AMPK mediates the activation of eEF2K, which in turn phosphorylates eEF2, leading to a decrease in translation elongation rates. Subsequently, eEF2K autophosphorylation generates a phosphodegron for the recruitment of the SCFβTrCP ubiquitin ligase. This event triggers the ubiquitylation of eEF2K and its proteasome-mediated degradation, which releases the inhibitory effect on eEF2 and translation elongation (see Figure 3). Regulation of translation elongation in response to DNA damage might have several advantages over controlling translation initiation. Indeed, elongation inhibition during checkpoint activation avoids the disassembly of polysomes, which, by stalling ribosomes on the mRNAs, might allow for mRNA protection from degradation or sequestration into stress granules. This mechanism will also ensure that translation can rapidly resume when the checkpoint is turned off. SCFβTrCP is implicated in the degradation of several substrates during the recovery from DNA damage and replication stress checkpoints. Indeed, βTrCP is required to reactivate the cyclin-dependent kinase Cdk1 by targeting the Cdk1 inhibitors Claspin and Wee1 for proteasome-dependent degradation and turn off the DNA repair machinery by causing the destruction of the Fanconi anemia protein FANCM. Our findings show that βTrCP is also needed during checkpoint silencing to resume protein synthesis by triggering proteolysis of eEF2K, suggesting that the ubiquitin ligase SCFβTrCP coordinates different processes (such as cell cycle progression, DNA repair, and protein synthesis) that are critical for checkpoint termination.

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Research team

summary

The kinase eEF2K controls the rate of peptide chain elongation by phosphorylating eEF2, the protein that mediates the movement of the ribosome along the mRNA by promoting translocation of the transfer RNA from the A to the P site in the ribosome. eEF2K-mediated phosphorylation of eEF2 on threonine 56 (Thr56) decreases its affinity for the ribosome, thereby inhibiting elongation. Here we show that in response to genotoxic stress, eEF2K was activated by AMPK (adenosine monophosphate-activated protein kinase)-mediated phosphorylation on serine 398. Activated eEF2K phosphorylated eEF2 and induced a temporary ribosomal slowdown at the stage of elongation. Subsequently, during DNA damage checkpoint silencing, a process required to allow cell cycle reentry, eEF2K was degraded by the ubiquitin-proteasome system through the ubiquitin ligase SCFβTrCP (Skp1-Cul1-F-box protein, β-transducin repeat-containing protein) to enable rapid resumption of translation elongation. This event required autophosphorylation of eEF2K on a canonical βTrCP-binding domain. The inability to degrade eEF2K during checkpoint silencing caused sustained phosphorylation of eEF2 on Thr56 and delayed the resumption of translation elongation. Our study therefore establishes a link between DNA damage signalling and translation elongation.
From November 15th to December 6th 2012 the bio-art project Blighted by Kenning is showcased at Hôtel Droog, Amsterdam. The Netherlands Proteomics Centre (NPC) and the British artist Charlotte Jarvis worked for almost a year on the project, in which apples grown at the Hague were ‘contaminated’ with synthetic DNA encoded with the Universal Declaration of Human Rights. Martje Ebberink, communications manager at the NPC, tells us more about the realisation of the project, the collaboration between Charlotte Jarvis and the scientists, and the hurdles they had to overcome.

Martje Ebberink remembers it as if it were yesterday. She explains: "The 6th of December 2011 was the turning point for Blighted by Kenning. Although remarkably enough, it was also the day we lost the Designers & Artists for Genomics Awards (DA4GA) contest. This inventive competition brings together elements from life sciences, art and design with the aim of bridging the gap between the life sciences and creative industries." During the so-called ‘speed dates’ scientists from leading genomics institutes, artists and designers were introduced to each other. Genomics centres and artists that were a ‘match’ were then asked to submit a proposal. On the last day of the contest, seven finalists presented their collaborative ideas to a professional jury from the fields of art and science. "From the moment we met Charlotte, we knew this could be the start of an innovative relationship. We strongly believe in Charlotte and the project,” states Ebberink. “After all the enthusiasm and positive reactions from professionals in the arts and sciences, we were disappointed that we were not one of the prize winners. We certainly would have been pleased to bring the project to life with a budget of €25.000 and showcase it at the museum Naturalis in Leiden.” The NPC found Charlotte’s idea so inspiring that they decided to make it a reality without the help of the DA4GA, and named her resident artist. From then on things moved very quickly.

Artistically and scientifically unique
After much preparation, Charlotte presented her vision at the NPC Progress Meeting in February 2012. Scientists at the NPC then went to work on bio-engineering a bacteria so that The Universal Declaration of Human Rights would be encoded into its DNA. The synthetic DNA was extracted from the bacteria.

On August 4, 2012 the grand opening of the Blighted by Kenning exhibit was held in a former dairy warehouse on the coast of Suffolk (UK) where a small apple orchard of thirteen trees was installed.
Life sciences join forces with art & design

The project is essentially a performance art piece in which an idea is biologically and literally spread across the world. It is artistically and scientifically unique in that it will create an international network of genomics institutes and a meaningful message by spreading a genetically engineered material. "Our hope is that Blighted by Kenning will contribute to the understanding of how information is encoded and expressed within DNA. We want to encourage people to think about what kind of information is contained within genetic material and how this can be manipulated. By creating a network of international institutions that contribute to the project, a statement will be made about the importance of genomics research and the interaction between science, technology and society,” says Ebberink.

Getting the licence

While working on the project, Charlotte Jarvis and the NPC encountered several obstacles. Ebberink explains: "At first we wanted to use the bacteria itself to contaminate the apples, not just its DNA. After consulting scientists at the NPC, some companies and a science museum, it was anticipated that it was nearly impossible to legally exhibit a Genetically Modified Organism (GMO) in a gallery.” Reinout Raijmakers, Managing Director of the Bijvoet Centre of Utrecht University, who served as a consultant for the scientific part of the project, came up with the idea to extract the DNA from the bacteria (leaving ‘naked DNA’ which does not carry the environmental risks associated with GMOs) and use this inert material to contaminate the apples. "Using naked DNA allowed us more freedom that might not otherwise be possible. For instance, we wanted to be able to ask people to eat the apples. Eating the apple allows scientists to make a gesture in support of genetics research,” Ebberink argues.

The decision was made to use the naked DNA, but things took longer than expected to get started. The most difficult part of the project was getting the licence to make the bacteria. She explains: "It turns out that we were the first group in the Netherlands to suggest encoding a fully synthetic protein sequence in DNA. Consequently, we were required to provide additional safety measures in order to obtain a license to make the DNA sequence.” After a couple of months, the NPC finally received notice that the permit was approved, and the apples were produced and mailed off to different corners of the earth.

Orchard inside the gallery

On August 4, 2012 the grand opening of the Blighted by Kenning exhibit was held in a former dairy warehouse on the coast of Suffolk (UK) where a small apple orchard of thirteen trees was installed. Ebberink reports: “Hanging on one of the trees was a sample of our forbidden fruit. There was also a billboard set up in the space, displaying images of The Declaration of Human Rights expressed as a protein. Films of the scientists eating the fruit, documents sent by institutes after sequencing the apples and a wall of correspondence detailing the making of the project were included in the exhibit.” During the opening ceremony Charlotte Jarvis ate one of the forbidden fruits. The next installment of the exhibit at Hotel Droog in Amsterdam will be more or less the same, but will include a documentary of the project.

Round table events were organised at both locations to question the current state of play with regards to popular perceptions of genetics. The topics were introduced by a number of speakers from science, the press and the arts, with an open floor discussion to close out the debate.
Valorisation

NPC Valorisation Voucher in practice

Maximum exploitation requires clear agreements

Researchers at Utrecht University and PamGene solved the problem of detecting phosphatase activity with a new peptide microarray. With the NPC Valorisation Voucher they were able to make the step towards a commercial product. "The success is chiefly based on the fruitful cooperation between university and company," assert the researchers involved.

In November 2011 Jeroen van Ameijde came up with a luminous idea, started a collaboration with PamGene and was also awarded an NPC Valorisation Voucher. One year later we talked to Jeroen van Ameide and Rob Ruijtenbeek, who look back on the effective progress of their commercialisation of a microarray technology for proteomics. "Now the validation phase has been successfully finished, we plan to offer the assay to a first batch of clients," they say. "That is a big advantage of working with a company that has a network of prospective clients," explains Jeroen Van Ameijde from the Medicinal Chemistry and Chemical Biology group at Utrecht University. "We as university researchers will help solve unexpected hurdles, but it is up to PamGene to commercialise the assay further."

Van Ameijde is very pleased with the successful collaboration. "Our groups have worked closely together for a long time. Publishing scientific results, which can be a controversial issue, has never been a problem since we have clear agreements with PamGene. So if relevant, the patent can be filed on time. But it is an issue that is always there," Van Ameijde adds. "When you regulate this topic by setting terms for publishing and filing patents at the start of a new collaboration there shouldn’t be a problem at all," he advises.

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Discovery and development

Kinases and phosphatases play a key role in signal transduction pathways in the cell. Together they regulate the phosphorylation patterns by respectively attaching and removing phosphate groups to and from proteins. "How the kinase enzyme family works and how we can intervene with drugs has been a subject of research for decades," explains van Ameijde. "There are very good tools available for studying and profiling kinase activity. Similar assays for researching phosphatases are still lacking." Knowledge about both enzyme families is important, since even a single disruption in phosphorylation patterns can cause a wide range of diseases, such as cancer. Van Ameijde: "The problem with measuring phosphatase activity is that the resulting molecule is hard to distinguish from other molecules in the cell because it has lost its phosphate label."

Together with Rob Ruijtenbeek of PamGene, the company that produces microarray assays for functional readouts of kinase and nuclear receptor activities, Van Ameijde had a luminous idea. What if they added an extra label to the substrate molecule, which would give a detectable product when the phosphatase is active. "It was a risky idea," he says. "In nature things work very specifically. So if you add even a small chemical group to a molecule, it can lose its interaction with the enzyme, especially when the label is near the place where phosphatases are active. To our surprise we saw it did not bother them at all."

Towards a real product

In the first test series they used about eight substrates. For a real product, dozens if not hundreds of substrates on the spots of a microarray are needed to make valuable phosphatase profiles. Van Ameijde: "We used the NPC Valorisation Voucher to scale it up and to demonstrate its effectiveness even in complex biological samples, and thereby take a step towards a real product." They also tested different situations that might be interesting for future customers. For instance, a company that has developed a new drug to inhibit a certain phosphatase would like to know how selective it is and whether it blocks other important enzymes too. "That is a huge problem with kinase inhibitors and has attracted the most attention. These kinds of tools are therefore extremely essential."
NPC theme leader Huib Ovaa and NPC project leader Sjaak Neefjes (Netherlands Cancer Institute) have been awarded an NPC Valorisation Voucher for their proposal 'Identification of the methotrexome for drug development'. Methotrexate is an agent used in cancer therapy, but is also effective in the treatment of rheumatoid arthritis and other autoimmune diseases. Ovaa and Neefjes are exploring a new target that explains the effects of methotrexate on autoimmunity, and chemical proteomics plays a central role in their approach. They aim to develop better medication without the side effects associated with methotrexate treatment. With this valorisation voucher the Neefjes and Ovaa labs target novel agents for rheumatoid arthritis therapy to be further developed in a novel startup company.

NPC Valorisation Voucher for Huib Ovaa and Sjaak Neefjes

No prognostic protein markers are currently available in the clinic for triple negative breast cancer. The newly developed protein profile has been generated with a dedicated state-of-the-art proteomics platform and provides an in-depth proteome analysis of minute amounts of clinical sample. The profile has recently been validated in an independent multi-centre patient cohort using the same proteome analysis. At present, quantitative, targeted mass spectrometry assays are being developed that should enable translation to the clinic.

Triple negative
Breast cancer affects one in eight women throughout their lifetimes and accounts for about 485,000 deaths annually. These numbers will increase by 13%, up to 548,000 incidences in 2019 (Datamonitor 2010). Most breast cancers are ER, PR or HER2 positive and can be effectively treated with targeted therapies directed against these proteins, such as hormonal therapies blocking production or function of estrogens, and antibody or kinase inhibitor therapies blocking the HER2 pathway. A minority, 10-15%, of breast cancers lack the three important clinical markers ER, PR and HER2 and are classified as triple negative (TN). Patients with TN breast cancer have an overall poor prognosis due to the aggressive nature of these tumours and lack of suitable targets for therapy. As a consequence, patients receive harsh chemotherapy that may display severe side effects and may be very debilitating. Of all patients with TN breast cancer, only 25% will develop distant metastases within five years after surgery. Therefore, the TN breast cancer can be divided into a good and poor prognostic group. However, as there is no effective prognostic screening method, patients with long-term progression free survival are also receiving harsh chemotherapy. Predicting beforehand which patients have favourable disease outcome and thus do not have to be treated with harsh chemotherapy would greatly improve the quality of life for these patients and reduce health care costs. In addition, markers for poor prognosis could be used as new targets against which new therapies for TN breast cancer can be developed.

Call for interested parties
A patent application has been filed and Erasmus MC is looking for a partner for a license to the technology with potential research collaboration. Interested parties are cordially invited to contact the Erasmus MC TTO to investigate the opportunities for licensing and/or research collaboration.

Contact information: Louise Hoppel, Business Developer, Technology Transfer Office, Erasmus MC, Rotterdam. (l.hoppel@erasmusmc.nl / +31 10 704 3343)

Novel biomarkers patent filed

Arzu Umar, NPC project leader for breast cancer proteomics at Erasmus MC, has found novel biomarkers for determining the prognosis of triple negative breast cancer. The prognostic profile helps defining the group of patients that are cured by surgery and prevents unnecessary treatment with chemotherapy. In addition, these biomarkers may serve as targets for development of novel therapies for triple negative breast cancer. A patent has recently been filed.

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Ron Heeren co-founder of Omics2Image B.V.

The start-up company Omics2Image has recently been established to bring technological innovations from the AMOLF group, lead by Prof. Ron Heeren, to the market. The first product of the start-up is the IonPix camera. This camera in combination with a microscope mode mass spectrometry system provides extremely high spatial resolution molecular images and has the capability of capturing several ion masses in one measurement cycle. The AMOLF technostarter Omics2Image works closely with Amsterdam Scientific Instruments, which focuses on other areas of detector technology. Omics2Image is the 3rd high-tech spin-off under the aegis of holding company Particle Physics Inside Products (P2IP). P2IP helps to navigate seed and early stage companies through the valorisation process, i.e. feasibility studies and business development.

NPC Valorisation Voucher for Huib Ovaa and Sjaak Neefjes

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Valorisation for scientists

What is valorisation?
Utilise knowledge to create social and economic value

By means of:
- Knowledge transfer to any interested party
- Collaboration (public/private)
- Protection of intellectual property
- Licensing
- Non-scientific publications / presentations

Towards
- New products, services, processes
- Spin-out company
- Education
- Publicity

I have an idea, what’s next?

Check if it needs protection
- Trade name
- Intellectual property
- Patent application

Contact TTO
For intellectual property, contracts, licensing, business development, brainstorm and much more, depending on TTO

Contact NPC
For brainstorm, advice, industry contacts, funding, business courses, business plans

What’s in it for me?

Why?
- Acknowledgement
- New sources of funding
- Knowledge dissemination
- Career development
- Your work understood and used by others

Why now?
- Anticipate on changes in research-climate
- Important criterium for publicly funded research-projects

Arrange funding
Check our website
www.netherlandsproteomicscentre.nl/funding/

Get coaching & advice

Check it out
www.netherlandsproteomicscentre.nl/IP/
www.lifesciencesatwork.nl

For more information please contact Adinda Woelderink
E-mail: woelderink@npc.genomics.nl
Phone: +31 (0)30 25 36 803
Many NPC projects are coming to an end. The PhD students involved are about to be released into the job market. Many of them are not sure what to do after graduation, as became clear when application for the BioBusiness Summer School 2012 was opened. “People ask me where I want to do my postdoc when they should be asking me what I want to do after I obtain my PhD,” says Adja Zoumaro, a PhD student at the Biomolecular Mass Spectrometry and Proteomics group in Utrecht. “I am not really interested in staying at the university as a postdoc, but I didn’t really know what else was out there. That is why I went to the Summer School," explains Adja. Benjamin Barasa, a PhD student working in the same group at Utrecht, says he hasn’t decided whether he wants to stay in academia or switch to industry. “It is fifty-fifty, but the Summer School gave the business side more weight.” Benjamin says he enjoyed the lecture by a board member of a large company who talked about the development of a drug for a rare disease. “This company has direct interaction with the patient. They develop these medicines even though the market is too small to make a profit. I was impressed by that." Adja found that a large portion of the Summer School was dedicated to people who want to start their own company. “But even though I am not interested in starting my own company, I learned a lot too. For example, during the session on business models, different steps from development to product in a typical biotech company were explained. I learned what type of job positions would be available to me in such a company.” Pepijn Burgers enjoyed the round table discussions which helped him decide what he wants to do in approximately one year when he finishes his PhD with the Mass Spec group at Utrecht. “The last year of a PhD student is often very busy; there is not much time to look ahead. So I was happy to go to the Summer School this year. The round table discussions convinced me that I would be able to pursue an academic career first in order to build up experience and then switch to industry.”

BioBusiness Summer School 2012 introduces prospective job-seekers to ‘the business world of Life Sciences’. Last June as many as 70 PhD students attended the week long Summer School in Amsterdam to hear about opportunities at large companies, possibilities for starting a business and options for extending their network.

BioBusiness Summer School 2012 informed Masters and PhD students and postdocs about career opportunities at one of the many Life Sciences companies during the five-day intensive programme (25-29 June). Inspiring speakers, professional trainers and guest subject specialists gave the lectures. Participants were informed about important business topics, had ample networking opportunities and met many representatives from leading Life Sciences companies. The BioBusiness Summer School will be organised again in 2013. Information on the date and possible sponsoring for participation will be announced on NPC’s website (www.netherlandsproteomicscentre.nl).

Career in Life Sciences

Benjamin Barasa, Adja Zoumaro and Pepijn Burgers participated in the BioBusiness Summer School 2012. “It is good to know what is out there.”

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Starting point

The students do not have set plans for more career orientation. Pepijn says that he will attend workshops if they come along. Adja, who is almost finished with her PhD, is the busiest. She is updating her CV and contacting people. “The Summer School was a very helpful starting point. I think this type of Summer School should be available much earlier in the course of study to prevent people from following the logical path of bachelor, master, PhD, postdoc without thinking it over.” Benjamin agrees: “I would recommend attending the Summer School to anyone, especially PhD students from Utrecht. I get the impression that people from other universities are more exposed to information regarding the opportunities for working in an industrial environment. It is good to hear about what else there is besides academia.”
First, a quick recap for those who are unfamiliar with the structure of DTL and what it envisions. Driven by the increasingly complex nature of life sciences research, a number of advanced technology centres and platforms in the Netherlands are building a new collaborative environment: the Dutch Techcentre for the Life Sciences. DTL offers scientists (in academia, medical centres and industry) the opportunity to tackle questions that require the use of multiple technologies, without having to address the different technology platforms separately. Ruben Kok elucidates: “Biologists increasingly look for ways to tackle the complexity of living systems on a new level. What they need is an integrative biology approach, where cross-links are established between technology disciplines. With DTL, we are creating an interface between the scientist and the various technology platforms and infrastructures for integrating and analysing big data.”

Cross-technology research
To date, DTL covers genomics/next generation sequencing, proteomics, metabolomics, advanced bioimaging as experimental technologies, systems biology, e-science and bioinformatics on the data handling and modelling side. It is no surprise that the Netherlands Proteomics Centre is the ‘proteomics-supplier’ of DTL. But there is more. NPC was the first to pioneer the concept of research hotels, and this successful concept will be continued at DTL. “When looking at the structure of DTL, most participating centres can be viewed as ‘research hotels’, local facilities that focus on a specific discipline, such as proteomics or bio-imaging. We are now building a network in which these individual hotels are connected to each other to create a cross-technology research facility. The ultimate goal is to create an environment where a biologist can present a research question or experiment that requires the use of multiple technologies and he or she is subsequently offered high-class expertise and facilities, like in a top-end hotel, to perform those experiments ‘in one go’. The ‘guest’ only has to address one ‘desk’, that being DTL.”

Big Data infrastructure
Significantly, the DTL collaboration does not stop when the data are generated. A key element of DTL is DISC, which stands for Data Integration and Stewardship Centre. Kok elaborates: “Each DTL facility is connected in DISC to ascertain the best integration and handing of the experimental data and other sources of relevant information. All the different technologies relating to data processing, storage and analysis come together here to form a broadly accessible Big Data infrastructure.” DISC is also the Dutch ‘node’ in ELIXIR, the European Life Sciences Infrastructure for Biological Information. “Being associated with international initiatives like ELIXIR is crucial for the future of life sciences research in the Netherlands,” says Kok, “that is why collaborating in entities like DTL is important for individual groups and technology centres. If we want to benefit from international programmes and, even better, influence the set-up and priorities of such initiatives, we have to be able to make a strong case. And that requires critical mass, as the participants in DTL clearly recognise.” He emphasises that DTL is open to all kinds of parties, whether their focus is on drug development, bio-based materials, equipment manufacturing, food, computational science — the list of potential topics is virtually endless. Kok concludes: “DTL welcomes anyone who operates at the forefront of life sciences.”

More information on DTL and DISC: www.dtls.nl
"Sustainability is a leading principle in managing our projects," says Marcel Wubbolts, Chief Technology Officer (CTO) at DSM and member of the NPC Supervisory Board. Wubbolts is responsible for overall technology for DSM and that includes the development of sustainable production methods for pharma, nutritional and materials applications. Wubbolts explains: "Our products include biobased materials for cars, biofuels from non-food feedstock and enzymes for medicine production. Sustainable production lowers energy and material use enormously. For example, initially the production of Cephalexin, a semi-synthetic cephalosporin, from Penicillin G required thirteen steps. We can now do it in three steps: first fermentation using a fungus in which engineered genes were introduced, followed by two enzymatic steps, all in water. The use of material and energy was reduced by 65 percent." Wubbolts stresses that proteomics techniques are essential for diverse applications such as quality control of production strains and enzymes. "When you introduce new genes into a fungus to improve a production process, you need to know if the enzymes reach the proper location within the fungus and whether they perform the way they should. That the fungus makes the desired product also needs to be proved. So we use techniques like mass spectrometry, NMR, 2-D gel electrophoresis and crystallography."

Collaboration with universities and institutes such as NPC plays an important role in the product development of DSM. "The initial phase of trying a new technology is often risky and costly. Working in consortiums divides the risks and costs. When we ascertain that a technique is strategically important via such a partnership, we also build it up in house. We have learned a great deal from universities this way and will continue to do so."

The NPC Supervisory Board represents the highest 'level of control'. The board advises the Executive Board on all matters related to the overall strategy and has to approve its main decisions. The Supervisory Board has final responsibility for all NPC activities. The Supervisory Board meets twice a year, with additional meetings if necessary. Marcel Wubbolts (DSM) and René Medema (Netherlands Cancer Institute) both joined the NPC Supervisory Board last year. They explain the importance of proteomics in current life sciences research.

Proteomics in the development of sustainable production

"Proteomics plays a very important role in oncology," affirms Prof. René Medema, chairman of the Board of the Netherlands Cancer Institute (NKI-AVL) and member of the NPC Supervisory Board. "Advances in both genomics and proteomics will enable the transition to personalised medicine. Thanks to these omics technologies, the diversity amongst tumours can be discerned. Proteomics enables us to discover biomarkers with which one can follow tumours, for example to detect whether they respond to a certain therapy or not. Proteomics is indispensable as a tool to unravel the fundamentals of oncobiology." Medema, who is also member of the NPC Supervisory board, is very glad to have access to new proteomics techniques through the NPC. "As a hospital one could decide to develop all the proteomics techniques in-house, but this would require enormous investments in people and equipment. However, as a user of the technologies you do want to have access to the latest innovations, so we are very glad that the NPC connects us to research groups that want to bring these technologies to an even higher level."

Medema stresses that the right infrastructure has proven vital to the innovative cancer research with which the NKI has built its international reputation. The improvement of cancer therapies and effective use of available medication have also profited from this infrastructure. Thanks to the NPC, proteomics research in the Netherlands is ideally organised, whereby developments are quickly passed on to other researchers."
Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors


Hubrecht Institute, KNAW and University Medical Center Utrecht, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

LGR5+ stem cells reside at crypt bottoms, intermingled with Paneth cells that provide Wnt, Notch and epidermal growth factor signals. Here we find that the related RNF43 and ZNRF3 transmembrane E3 ubiquitin ligases are uniquely expressed in LGR5+ stem cells. Simultaneous deletion of the two genes encoding these proteins in the intestinal epithelium of mice induces rapidly growing adenomas containing high numbers of Paneth and LGR5+ stem cells. In vitro, growth of organoids derived from these adenomas is arrested when Wnt secretion is inhibited, indicating a dependence of the adenoma stem cells on Wnt produced by adenoma Paneth cells. In the HEK293T human cancer cell line, expression of RNF43 blocks Wnt responses and targets surface-expressed frizzled receptors to lysosomes. In the RNF43-mutant colorectal cancer cell line HCT116, reconstitution of RNF43 expression removes its response to exogenous Wnt. We conclude that RNF43 and ZNRF3 reduce Wnt signals by selectively ubiquitinating frizzled receptors, thereby targeting these Wnt receptors for degradation.

The landscape of cancer genes and mutational processes in breast cancer

Stephens, P.J. et. al.
Cancer Genome Project, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton CB10 1SA, UK.

All cancers carry somatic mutations in their genomes. A subset, known as driver mutations, confer clonal selective advantage on cancer cells and are causally implicated in oncogenesis, and the remainder are passenger mutations. The driver mutations and mutational processes operative in breast cancer have not yet been comprehensively explored. Here we examine the genomes of 100 tumours for somatic copy number changes and mutations in the coding exons of protein-coding genes. The number of somatic mutations varied markedly between individual tumours. We found strong correlations between mutation number, age at which cancer was diagnosed and cancer histological grade, and observed multiple mutational signatures, including one present in about ten per cent of tumours characterized by numerous mutations of cytosine at TpC dinucleotides. Driver mutations were identified in several new cancer genes including AKT2, ARID1B, CASP8, CDKN1B, MAP3K1, MAP3K13, NCOA1, SMARCD1 and TBX3. Among the 100 tumours, we found driver mutations in at least 40 cancer genes and 73 different combinations of mutated cancer genes. The results highlight the substantial genetic diversity underlying this common disease.
The life history of 21 breast cancers

Nik-Zainal, S., et al. Cancer Genome Project, Wellcome Trust Sanger Institute, Hinxton CB10 1SA, UK.

Cancer evolves dynamically as clonal expansions supersed one another driven by shifting selective pressures, mutational processes, and disrupted cancer genes. These processes mark the genome, such that a cancer’s life history is encrypted in the somatic mutations present. We developed algorithms to decipher this narrative and applied them to 21 breast cancers. Mutational processes evolve across a cancer’s lifespan, with many emerging late but contributing extensive genetic variation. Subclonal diversification is prominent, and most mutations are found in just a fraction of tumor cells. Every tumor has a dominant subclonal lineage, representing more than 50% of tumor cells. Minimal expansion of these subclones occurs until many hundreds to thousands of mutations have accumulated, implying the existence of long-lived, quiescent cell lineages capable of substantial proliferation upon acquisition of enabling genomic changes. Expansion of the dominant subclone to an appreciable mass may therefore represent the final rate-limiting step in a breast cancer’s development, triggering diagnosis.

Triple bioorthogonal ligation strategy for simultaneous labeling of multiple enzymatic activities


Three at the same time: A ligation strategy combining tetra-zine-norbornene cycloaddition, Staudinger-Bertozzi ligation, and copper(I)-catalyzed click reaction was used to label the three catalytic activities of the proteasome simultaneously in a single experiment. The orthogonality of the three ligation reactions enables selective monitoring of multiple targets at the same time in complex biological samples.

A key role for Chd1 in histone H3 dynamics at the 3’ ends of long genes in yeast


Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

Chd proteins are ATP-dependent chromatin remodeling enzymes implicated in biological functions from transcriptional elongation to control of pluripotency. Previous studies of the Chd1 subclass of these proteins have implicated them in diverse roles in gene expression including functions during initiation, elongation, and termination. Furthermore, some evidence has suggested a role for Chd1 in replication-independent histone exchange or assembly. Here, we examine roles of Chd1 in replication-independent dynamics of histone H3 in both Drosophila and yeast. We find evidence of a role for Chd1 in H3 dynamics in both organisms. Using genome-wide ChIP-on-chip analysis, we find that Chd1 influences histone turnover at the 5’ and 3’ ends of genes, accelerating H3 replacement at the 5’ ends of genes while protecting the 3’ ends of genes from excessive H3 turnover. Although consistent with a direct role for Chd1 in exchange, these results may indicate that Chd1 stabilizes nucleosomes perturbed by transcription. Curiously, we observe a strong effect of gene length on Chd1’s effects on H3 turnover. Finally, we show that Chd1 also affects histone modification patterns over genes, likely as a consequence of its effects on histone replacement. Taken together, our results emphasize a role for Chd1 in histone replacement in both budding yeast and Drosophila melanogaster, and surprisingly they show that the major effects of Chd1 on turnover occur at the 3’ ends of genes.

An N-terminal acidic region of Sgs1 interacts with Rpa70 and recruits Rad53 kinase to stalled forks


Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland [2] Faculty of Sciences, University of Basel, Basel, Switzerland...
The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent ‘+4’ cell markers


Bijvoet Center for Biomolecular Research, Utrecht Institute for Pharmaceutical Sciences, University, The Netherlands.

Two types of stem cells are currently defined in small intestinal crypts: cycling crypt base columnar (CBC) cells and quiescent ‘+4’ cells. Here, we combine transcriptomics with proteomics to define a definitive molecular signature for Lgr5(+) CBC cells. Transcriptional profiling of FACS-sorted Lgr5(+) stem cells and their daughters using two microarray platforms revealed an mRNA stem cell signature of 384 unique genes. Quantitative mass spectrometry on the same cell populations identified 278 proteins enriched in intestinal stem cells. The mRNA and protein data sets showed a high level of correlation and a combined signature of 510 stem cell-enriched genes was defined. Spatial expression patterns were further characterized by mRNA in-situ hybridization, revealing that approximately half of the genes were expressed in a gradient with highest levels at the crypt bottom, while the other half was expressed uniquely in Lgr5(+) stem cells. Lineage tracing using a newly established knock-in mouse for one of the signature genes, Smoc2, confirmed its stem cell specificity. Using this resource, we find-and confirm by independent approaches—that the proposed quiescent ‘+4’ stem cell markers Bmi1, Tert, Hopx and Lrig1 are robustly expressed in CBC cells.

Other highlighted publications

Albers, H.M., Ovaa, H.

Chemical evolution of autotaxin inhibitors

Kruiswijk, F., Yuniati, L., Magliziozzi, R., Low, T.Y., Lim, R., Bolder, R., Mohammed, S., Proud, C.G., Heck, A.J., Pagano, M., Guardavaccaro, D.

Coupled activation and degradation of eEF2K regulates protein synthesis in response to genotoxic stress

Meinema, A.C., Poolman, B., Veenhoff, L.M.

The transport of integral membrane proteins across the nuclear pore complex

Ongay S., Boichenko A., Govorukhina N., Bischoff R.

Glycopeptide enrichment and separation for protein glycosylation analysis

Bischoff, R., and Schlüter, H.

Amino acids: Chemistry, functionality and selected non-enzymatic post-translational modifications
J. Proteomics (2012) 75, 2275-2296

Kovanich, D., Aye, T.T., Heck, A.J., Scholten, A.

Probing the specificity of protein-protein interactions by quantitative chemical proteomics

Kovanich, D, Cappadona, S., Rajmakers, R., Mohammed, S., Scholten, A., Heck, A.J.

Applications of stable isotope dimethyl labeling in quantitative proteomics

van Geel, R., Pruijn, G.J., van Delft, F.L., Boelens, W.C.

Preventing thiol-yne addition improves the specificity of strain-promoted azide-alkyne cycloaddition


Spatially Resolving the Secretome within the Mycelium of the Cell Factory Aspergillus niger


Cofactor Binding Protects Flavodoxin against Oxidative Stress

Smaczniak, C., Immink, R.G., Angenent, G.C., Kaufmann, K.

Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies


Validation of a radiosensitivity molecular signature in breast cancer


BCAR4 induces antiestrogen resistance but sensitizes breast cancer to lapatinib

Richau, K.H., Kaschani, F., Verdoes, M., Pansuriya, T.C., Niessen, S., Stüker, K., Colby, T., Overkleeft, H.S., Bogoy, M., Van der Hoorn, R.A.

Subclassification and biochemical analysis of plant papain-like cysteine proteases displays subfamily-specific characteristics.
10 Years NPC: Progress in Proteome Biology

The meeting is dedicated to the 10th anniversary of the NPC. In a special programme outstanding national and international scientists will present their views on the newest developments in proteomics with lectures, NPC project posters and an exhibition. The celebration will be completed with the NPC Anniversary Dinner on February 11th.

We look forward to seeing you at this anniversary meeting!

Registration & abstract deadline: 21 January 2013
www.netherlandsproteomicscentre.nl
progressmeeting@npc.genomics.nl

Confirmed speakers include
Albert Heck
Connie Jimenez
Roland Kanaar
Jeroen Krijgsveld
Madelon Maurice
René Medema
Christine Mummery
Maurien Olsthoorn
Bert Poolman
Carol Robinson
Ben Scheres
Marc Timmers
Mathias Uhlen
Arzu Umar
Golden future

In 2002, the Netherlands Genomics Initiative (NGI) was launched. Consequently, it was decided that the field of genomics could benefit from a solid infrastructure with a collaborative foundation. From this fundamental idea came the initiative taken by Albert Heck and his partners to put together the Netherlands Proteomics Centre (NPC) - a seamless and award-winning fit, backed by an excellent proposal.

When the first phase of the NPC began, in 2003, I had the privilege of serving as chairman of the Supervisory Board, and I can now proudly say that I have enjoyed the position for ten years. Fortunately, Albert Heck and his team have continuously succeeded in securing funds for a follow-up programme, starting with an award of 25 million euros, another estimating 22 million euros, and finally 13.5 million euros for 2014-2018. To further substantiate that our work has had a significant impact, knowledge institutes and the business community have continued to support the NPC by matching these funds.

Proteomics is a crucial part of Dutch life sciences, with many spin-offs, and thanks to the NPC, we can now say that the Netherlands is a major player in this field. For example, there are the countless scientific publications, the well-attended yearly progress meetings, and let’s not forget the successful HUPO in 2008 meeting which brought international protein researchers from all over the globe to the Netherlands.

On a larger scale, proteomics will undoubtedly make a major contribution to the understanding of biological processes at the protein level. This will lead to the development of new medicines and biomarkers that will have a huge impact on the diagnosis and treatment of diseases, but it will also affect other fields in the life sciences e.g. the agro-food sector.

The solid network built up in the Netherlands is the result of creativity and perseverance, but most notably the will to cooperate by sharing and delivering TOP results. Continued expansion in the coming years and the drive to lead activities in the Life Sciences and Health sector will prove to be highly productive for the Dutch life sciences, but most certainly also for the NPC. As chairman of the Supervisory Board I feel confident in passing on the torch to my successor, and reflect fondly on my years of contribution. I would like to thank Albert Heck, his team and partners, and also my fellow members of the Supervisory Board for a productive and pleasant working experience. I wish the NPC a golden future.

Eduard Klasen
Professor Emeritus
Dean & Member Executive Board LUMC till June 2012
Chairman NPC Executive Board 2003-2012