Exploring extracellular matrix degradomes by TMT-TAILS N-terminomics

Global characterization of protein N termini provides valuable information on proteome dynamics and diversity in health and disease. Driven by the progress in mass spectrometry-based proteomics, novel approaches for the dedicated investigation of protein N termini and protease substrates have been recently developed. Terminal amine isotopic labeling of substrates (TAILS) is a quantitative proteomics approach suitable for high-throughput and system-wide profiling of protein N termini in complex biological matrices. TAILS employs isotopic labeling of primary amines of intact proteins in combination with an amine-reactive high molecular weight polymer (HPG-ALD) for depletion of internal tryptic peptides and high enrichment of protein N termini by negative selection. Thereby, TAILS allows simultaneous identification of the natural N termini, protease-generated neo-N termini, and endogenously modified (e.g., acetylated) N termini. In this chapter, we provide a protocol for tandem mass tag (TMT)-TAILS analysis and further discuss specific considerations regarding N-terminome data interpretation using Proteome Discoverer™ software.

Mapping the N-Termimome in Tissue Biopsies by PCT-TAILS

Proteases play pivotal roles in multiple biological processes in all living organisms and are tightly regulated under normal conditions, but alterations in the proteolytic system and uncontrolled protease activity result in multiple pathological conditions. A disease will most often be defined by an ensemble of cleavage events—a proteolytic signature, thus the system-wide study of protease substrates has gained significant attention and identification of disease specific clusters of protease substrates holds great promise as targets for diagnostics and therapy. In this chapter we describe a method that enables fast and reproducible analysis of protease substrates and proteolytic products in an amount of tissue less than the quantity obtained by a standard biopsy. The method combines tissue disruption and protein extraction by pressure cycling technology (PCT), N-terminal enrichment by tandem mass tag (TMT)-terminal amine isotopic labeling of substrates (TAILS), peptide analysis by mass spectrometry (MS), and a general pipeline for interpretation of the data.
Post-translational modification-dependent activity of matrix metalloproteinases

Due to their capacity to process different proteins of the extracellular matrix (ECM), matrix metalloproteinases (MMPs) were initially described as a family of secreted proteases, functioning as main ECM regulators. However, through proteolytic processing of various biomolecules, MMPs also modulate intra- and extracellular pathways and networks. Thereby, they are functionally implicated in the regulation of multiple physiological and pathological processes. Consequently, MMP activity is tightly regulated through a combination of epigenetic, transcriptional, and post-transcriptional control of gene expression, proteolytic activation, post-translational modifications (PTMs), and extracellular inhibition. In addition, MMPs, their substrates and ECM binding partners are frequently modified by PTMs, which suggests an important role of PTMs in modulating the pleiotropic activities of these proteases. This review summarizes the recent progress towards understanding the role of PTMs (glycosylation, phosphorylation, glycosaminoglycans) on the activity of several members of the MMP family.
Protease activity profiling of snake venoms using high-throughput peptide screening

Snake venom metalloproteinases (SVMPs) and snake venom serine proteinases (SVSPs) are among the most abundant enzymes in many snake venoms, particularly among vipers. These proteinases are responsible for some of the clinical manifestations classically seen in viperid envenomings, including hemorrhage, necrosis, and coagulopathies. The objective of this study was to investigate the enzymatic activities of these proteins using a high-throughput peptide library to screen for the proteinase targets of the venoms of five viperid (Echis carinatus, Bothrops asper, Daboia russellii, Bitis arietans, Bitis gabonica) and one elapid (Naja nigricollis) species of high medical importance. The proteinase activities of these venoms were each tested against 360 peptide substrates, yielding 2160 activity profiles. A nonlinear regression model that accurately described the observed enzymatic activities was fitted to the experimental data, allowing for the comparison of cleavage rates across species. In this study, previously unknown protein targets of snake venom proteinases were identified, potentially implicating novel human and animal proteins that may be involved in the pathophysiology of viper envenomings. The functional relevance of these targets was further evaluated and discussed. These new findings may contribute to our understanding of the clinical manifestations and underlying biochemical mechanisms of snakebite envenoming by viperid species.

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A SYSTEM-WIDE STUDY OF THE PROTEOME AND PROTEASE CLEAVAGE PRODUCTS IN CHRONIC SKIN ULCERS

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Body Fluid Degradomics and Characterization of Basic N-Terminome

Rapid improvements in instrumentation and data analysis make mass spectrometry-based proteomics the method of choice for global characterization of proteomes and discovery of protein-based biomarkers. On the contrary to tissue biopsies, body fluids—e.g., blood, wound fluid, urine, and saliva—are noninvasive and easy to collect and process. However, they are very complex and present high dynamic ranges of protein concentrations, rendering direct shotgun proteomics analysis as inefficient for identification of low-abundance proteins in these specimens. Sample prefractionation, immunoaffinity depletion of highly abundant proteins, and enrichment of posttranslational modifications (PTM) are common strategies for proteome simplification of body fluids. Combinatorial peptide ligand libraries (CPLL) relatively deplete high-abundance proteins by binding equimolar amounts of protein species in the sample and provide an elegant species-independent alternative to immunoaffinity-based approaches. By cleaving target proteins, proteases catalyze an irreversible PTM, whereby uncontrolled proteolysis is associated with many diseases. Thus, proteolytic events represent powerful indicators for disease progression and their specific identification in body fluids holds great promises for establishment of novel biomarkers. Quantitative N-terminal enrichment strategies, such as terminal amine isotopic labeling of substrates (TAILS) detect protease-generated neo-N-termini with high specificity and increase coverage of low-abundance proteins by inherent proteome simplification. In this chapter, we describe a protocol that combines the CPLL technology with iTRAQ-based TAILS to systematically characterize the basic N-terminome of body fluid proteomes and its alterations in disease conditions that we have successfully applied to explore the wound fluid degradome at multiple time points after skin injury.

Comparative degradomics of porcine and human wound exudates unravels biomarker candidates for assessment of wound healing progression in trauma patients

Impaired cutaneous wound healing is a major complication in elderly people and patients suffering from diabetes with raising rates in industrialized countries. Heterogeneity of clinical manifestations hampers effective molecular diagnostics and decisions for appropriate therapeutic regimens. Using a customized positional quantitative proteomics workflow, we have established a time-resolved proteome and N-terminome resource from wound exudates in a clinically relevant pig wound model that we exploited as robust template to interpret a heterogeneous dataset from patients undergoing the same wound treatment. With zyxin, IQG1, and HtrA1, this analysis and validation by targeted proteomics identified differential abundances and proteolytic processing of proteins of epidermal and dermal origin as prospective biomarker candidates for assessment of critical turning points in wound progression. Thus, we demonstrate the possibility of using a fine-tuned animal wound model to bridge the translational gap as prerequisite for future extended clinical studies with large cohorts of individuals affected by healing impairments. Data are available via ProteomeXchange with identifier
Large-Scale Quantitative Proteomics Identifies the Ubiquitin Ligase Nedd4-1 as an Essential Regulator of Liver Regeneration

The liver is the only organ in mammals that fully regenerates even after major injury. To identify orchestrators of this regenerative response, we performed quantitative large-scale proteomics analysis of cytoplasmic and nuclear fractions from normal versus regenerating mouse liver. Proteins of the ubiquitin-proteasome pathway were rapidly upregulated after two-third hepatectomy, with the ubiquitin ligase Nedd4-1 being a top hit. In vivo knockdown of Nedd4-1 in hepatocytes through nanoparticle-mediated delivery of small interfering RNA caused severe liver damage and inhibition of cell proliferation after hepatectomy, resulting in liver failure. Mechanistically, we demonstrate that Nedd4-1 is required for efficient internalization of major growth factor receptors involved in liver regeneration and their downstream mitogenic signaling. These results highlight the power of large-scale proteomics to identify key players in liver regeneration and the importance of posttranslational regulation of growth factor signaling in this process. Finally, they identify an essential function of Nedd4-1 in tissue repair.
Multidimensional analysis of protease substrates and their cellular origins in mixed secretomes from multiple cell types

Although extracellular proteases are confronted with substrate proteins expressed by multiple cell types in vivo, in most protease substrate discovery approaches, the test protease is exposed to a test proteome (secretome) derived only from a single cell type. This limits the potential substrate space and prohibits the formation of protein complexes constituted of components derived from multiple cellular origins. Mixing of secretomes collected from multiple cell types addresses this issue, but information on the cellular origin of a substrate protein is lost. Here, we describe a protocol and the corresponding data analysis workflow for a multidimensional substrate discovery approach termed SILAC-iTRAQ-TAILS that is based on hyperplexed terminal amine isotopic labeling of substrates (TAILS), allowing identification of substrates and concomitant assignment to cellular origins in mixed secretomes within the same experiment.

Targeted degradomics in protein terminomics and protease substrate discovery

Targeted degradomics integrates positional information into mass spectrometry-based targeted proteomics workflows and thereby enables analysis of proteolytic cleavage events with unprecedented specificity and sensitivity. Rapid progress in establishment of protease-substrate relations provides extensive degradomics target lists that now can be tested with help of selected and parallel reaction monitoring (S/PRM) in complex biological systems, where proteases act in physiological environments. In this minireview, we describe the general principles of targeted degradomics, outline the generic experimental workflow of the methodology and highlight recent and future applications in protease research.
Time-resolved analysis of matrix metalloproteinase substrates in complex samples

Identification of physiological substrates is the key to understanding the pleiotropic functions of matrix metalloproteinases (MMPs) in health and disease. Quantitative mass spectrometry-based proteomics has revolutionized current approaches in protease substrate discovery and helped to unravel many new MMP activities in complex biological systems. Multiplexing further extended the capabilities of these techniques and facilitated more complicated experimental designs that include multiple proteases or monitoring the activity of a single protease at more than one concentration or at multiple time points with a complex test proteome. In this chapter, we provide a protocol for time-resolved iTRAQ-based Terminal Amine Isotopic Labeling of Substrates (TAILS), with the focus on MMP substrate identification and characterization in cell culture supernatants and introduce an automated procedure for the interpretation of time-resolved iTRAQ-TAILS datasets.

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Autocrine and paracrine regulation of keratinocyte proliferation through a novel nrf2-il-36γ pathway

The Nrf2 transcription factor is well known for its cytoprotective functions through regulation of genes involved in the detoxification of reactive oxygen species or toxic compounds. Therefore, activation of Nrf2 is a promising strategy for the protection of tissues from various types of insults and for cancer prevention. However, recent studies revealed a proinflammatory activity of activated Nrf2 and a stimulating effect on epithelial cell proliferation, but the underlying mechanisms of action and the responsible target genes are largely unknown. Using a combination of gene expression profiling, chromatin immunoprecipitation, and targeted proteomics via selected reaction monitoring, we show that the gene encoding the proinflammatory cytokine IL-36γ is a novel direct target of Nrf2 in keratinocytes and hepatocytes in vitro and in vivo. As a consequence, upregulation of IL-36γ expression occurred upon genetic or pharmacological activation of Nrf2 in the epidermis and in the normal and regenerating liver. Functional in vitro studies demonstrate that IL-36γ directly stimulates proliferation of keratinocytes. In particular, it induces expression of keratinocyte mitogens in fibroblasts, suggesting that the Nrf2-IL-36γ axis promotes keratinocyte proliferation through a double paracrine loop. These results provide mechanistic insight into Nrf2 action in the control of inflammation and cell proliferation through regulation of a proinflammatory cytokine with a key function in various inflammatory diseases.
TAILS N-Terminomics and Proteomics Show Protein Degradation Dominates over Proteolytic Processing by Cathepsins in Pancreatic Tumors

Deregulated cathepsin proteolysis occurs across numerous cancers, but in vivo substrates mediating tumorigenesis remain ill-defined. Applying 8-plex iTRAQ terminal amine isotopic labeling of substrates (TAILS), a systems-level N-terminome degradomics approach, we identified cathepsin B, H, L, S, and Z in vivo substrates and cleavage sites with the use of six different cathepsin knockout genotypes in the Rip1-Tag2 mouse model of pancreatic neuroendocrine tumorigenesis. Among 1,935 proteins and 1,114 N termini identified by TAILS, stable proteolytic products were identified in wild-type tumors compared with one or more different cathepsin knockouts (17%–44% of 139 cleavages). This suggests a lack of compensation at the substrate level by other cathepsins. The majority of neo-N termini (56%–83%) for all cathepsins was consistent with protein degradation. We validated substrates, including the glycolytic enzyme pyruvate kinase M2 associated with the Warburg effect, the ER chaperone GRP78, and the oncoprotein prothymosin-alpha. Thus, the identification of cathepsin substrates in tumorigenesis improves the understanding of cathepsin functions in normal physiology and cancer.
Active site specificity profiling of the matrix metalloproteinase family: Proteomic identification of 4300 cleavage sites by nine MMPs explored with structural and synthetic peptide cleavage analyses

Secreted and membrane tethered matrix metalloproteinases (MMPs) are key homeostatic proteases regulating the extracellular signaling and structural matrix environment of cells and tissues. For drug targeting of proteases, selectivity for individual molecules is highly desired and can be met by high yield active site specificity profiling. Using the high throughput Proteomic Identification of protease Cleavage Sites (PICS) method to simultaneously profile both the prime and non-prime sides of the cleavage sites of nine human MMPs, we identified more than 4300 cleavages from P6 to P6' in biologically diverse human peptide libraries. MMP specificity and kinetic efficiency were mainly guided by aliphatic and aromatic residues in P1' (with a ~32–93% preference for leucine depending on the MMP), and basic and small residues in P2' and P3', respectively. A wide differential preference for the hallmark P3 proline was found between MMPs ranging from 15 to 46%, yet when combined in the same peptide with the universally preferred P1' leucine, an unexpected negative cooperativity emerged. This was not observed in previous studies, probably due to the paucity of approaches that profile both the prime and non-prime sides together, and the masking of subsite cooperativity effects by global heat maps and iceLogos. These caveats make it critical to check for these biologically highly important effects by fixing all 20 amino acids one-by-one in the respective subsites and thorough assessing of the inferred specificity logo changes. Indeed an analysis of bona fide MEROPS physiological substrate cleavage data revealed that of the 37 natural substrates with either a P3-Pro or a P1'-Leu only 5 shared both features, confirming the PICS data. Upon probing with several new quenched-fluorescent peptides, rationally designed on our specificity data, the negative cooperativity was explained by reduced non-prime side flexibility constraining accommodation of the rigidifying P3 proline with leucine locked in S1'. Similar negative cooperativity between P3 proline and the novel preference for asparagine in P1 cements our conclusion that non-prime side flexibility greatly impacts MMP binding affinity and cleavage efficiency. Thus, unexpected sequence cooperativity consequences were revealed by PICS that uniquely encompasses both the non-prime and prime sides flanking the proteomic-pinpointed scissile bond.

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Caspase-1 activity is required for UVB-induced apoptosis of human keratinocytes
Caspase-1 has a crucial role in innate immunity as the protease activates the proinflammatory cytokine prointerleukin(IL)-1β. Furthermore, caspase-1 induces pyroptosis, a lytic form of cell death that supports inflammation. Activation of caspase-1 occurs in multi-protein complexes termed inflammasomes, which assemble upon sensing of stress signals. In the skin and in skin-derived keratinocytes, UVB irradiation induces inflammasome-dependent IL-1 secretion and sunburn. Here we present evidence that caspase-1 and caspase-4 are required for UVB-induced apoptosis. In UVB-irradiated human primary keratinocytes, apoptosis occurs significantly later than inflammasome activation but depends on caspase-1 activity. However, it proceeds independently of inflammasome activation. By a proteomics approach, we identified the antiapoptotic Bap31 as a putative caspase-1 substrate. Caspase-1-dependent apoptosis is possibly a recent process in evolution as it was not detected in mice. These results suggest a protective role of caspase-1 in keratinocytes during UVB-induced skin cancer development through the induction of apoptosis.

General information
Identification of protease substrates in complex proteomes by iTRAQ-TAILS on a thermo Q Exactive instrument

The human genome encodes more than 560 proteases, but only for few of them the substrate proteins are known. This is mainly due to high numbers of potential substrate targets for any given protease that cannot be comprehensively explored by conventional candidate approaches. In this chapter, we describe a proteomics protocol for the reliable identification of protease substrates on a proteome-wide scale. Notably, this method termed iTRAQ-Terminal Amine Isotopic Labeling of Substrates (TAILS) does not require any prior knowledge on candidate proteins. Instead, it is used as an unbiased discovery approach to identify protease-substrate relations in complex biological samples. In addition, iTRAQ-TAILS not only identifies substrate proteins but also maps cleavage sites with amino acid precision. Knowing the cleavage site enables the researcher to perform specific downstream analyses and eliminates the need for laborious follow-up experiments like Edman sequencing. iTRAQ-TAILS acquires this rich information by exploiting the power of latest generation mass spectrometers as the Thermo Q Exactive instrument. Through quantitative assessment of protein N-termini in protease-exposed and control samples and a robust data analysis pipeline, iTRAQ-TAILS can systematically screen proteome-wide substrate spaces for proteolytic events exerted by proteases of interest.

In vivo assessment of protease dynamics in cutaneous wound healing by degradomics analysis of porcine wound exudates

Proteases control complex tissue responses by modulating inflammation, cell proliferation and migration, and matrix remodeling. All these processes are orchestrated in cutaneous wound healing to restore the skin’s barrier function upon
Altered protease activity has been implicated in the pathogenesis of healing impairments, and proteases are important targets in diagnosis and therapy of this pathology. Global assessment of proteolysis at critical turning points after injury will define crucial events in acute healing that might be disturbed in healing disorders. As optimal biospecimens, wound exudates contain an ideal proteome to detect extracellular proteolytic events, are noninvasively accessible, and can be collected at multiple time points along the healing process from the same wound in the clinics. In this study, we applied multiplexed Terminal Amine Isotopic Labeling of Substrates (TAILS) to globally assess proteolysis in early phases of cutaneous wound healing. By quantitative analysis of proteins and protein N termini in wound fluids from a clinically relevant pig wound model, we identified more than 650 proteins and discerned major healing phases through distinctive abundance clustering of markers of inflammation, granulation tissue formation, and re-epithelialization. TAILS revealed a high degree of proteolysis at all time points after injury by detecting almost 1300 N-terminal peptides in ~450 proteins. Quantitative positional proteomics mapped pivotal interdependent processing events in the blood coagulation and complement cascades, temporally discerned clotting and fibrinolysis during the healing process, and detected processing of complement C3 at distinct time points after wounding and by different proteases. Exploiting data on primary cleavage specificities, we related candidate proteases to cleavage events and revealed processing of the integrin adapter protein kindlin-3 by caspase-3, generating new hypotheses for protease-substrate relations in the healing skin wound in vivo. The data have been deposited to the Proteome-Xchange Consortium with identifier PXD001198.
Matrix metalloproteinases in impaired wound healing

Cutaneous wound healing is a complex tissue response that requires a coordinated interplay of multiple cells in orchestrated biological processes to finally re-establish the skin's barrier function upon injury. Proteolytic enzymes and in particular matrix metalloproteinases (MMPs) contribute to all phases of the healing process by regulating immune cell influx, facilitating migration of fibroblasts and keratinocytes, and remodeling of the scar tissue. As a result of these pleiotropic functions in the healing skin wound, uncontrolled activities of MMPs are associated with impaired wound healing, a growing health problem in Western countries due to increased life expectancies and rising rates of underlying diseases, such as diabetes. However, detailed mechanisms have been only partially unraveled, and new diagnostic tools and more targeted therapies are urgently needed. In this review, we discuss the roles of MMPs in acute and chronic wound healing and summarize current therapeutic approaches aiming at inhibiting aberrant MMP activities in healing disorders.

MMPs: From Genomics to Degradomics

This chapter discusses how the drawbacks of a conventional genomics approach led to the misevaluation of matrix metalloproteinases (MMPs) as therapeutic targets. It summarizes recent advancements in our understanding of MMPs as master regulators of tissue homeostasis, and points to specific challenges of using MMPs as therapeutic targets. The chapter introduces the concepts of degradomics-based systems biology and demonstrates how its application to MMP research deepened our understanding of the interconnected activities of members of this protease class within the protease web. The chapter also reviews the results obtained from the genomics approach to MMP function in detail, pointing out valuable information as well as former missing links, those which eventually resulted in the misled predictions and expectations of the effects of therapeutic intervention with MMPs. Global assessment of MMP expression and, more importantly, activity will bring us closer to depicting complete MMP activity degradomes in complex systems.
Monitoring matrix metalloproteinase activity at the epidermal-dermal interface by SILAC-iTRAQ-TAILS

Secreted proteases act on interstitial tissue secretomes released from multiple cell types. Thus, substrate proteins might be part of higher molecular complexes constituted by many proteins with diverse and potentially unknown cellular origin. In cell culture, these may be reconstituted by mixing native secretomes from different cell types prior to incubation with a test protease. Although current degradomics techniques could identify novel substrate proteins in these complexes, all information on the cellular origin is lost. To address this limitation, we combined iTRAQ-based terminal amine isotopic labeling of substrates (iTRAQ-TAILS) with SILAC to assign proteins to a specific cell type by MS1- and their cleavage by MS2-based quantification in the same experiment. We demonstrate the power of our newly established workflow by monitoring matrix metalloproteinase (MMP) 10 dependent cleavages in mixtures from light-labeled keratinocyte and heavy-labeled fibroblast secretomes. This analysis correctly assigned extracellular matrix components, such as laminins and collagens, to their respective cellular origins and revealed their processing in an MMP10-dependent manner. Hence, our newly devised degradomics workflow facilitates deeper insight into protease activity in complex intercellular compartments such as the epidermal-dermal interface by integrating multiple modes of quantification with positional proteomics. All MS data have been deposited in the ProteomeXchange with identifier PXD001643.

Proteomics approaches to uncover MMP function

Proteomics has revolutionized protease research and particularly contributed to the identification of novel substrates and their sites of cleavage as key determinants of protease function. New technologies and rapid advancements in development of powerful mass spectrometers allowed unprecedented insights into activities of matrix metalloproteinases (MMPs) within their complex extracellular environments. Mass spectrometry-based proteomics extended our knowledge...
on MMP cleavage specificities and will help to develop more specific inhibitors as new therapeutics. Quantitative proteomics and N-terminal enrichment strategies have revealed numerous novel MMP substrates and shed light on their modes of action in vitro and in vivo. In this review, we provide an overview of current proteomic technologies in protease research and their application to the functional characterization of MMPs.

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**Macrophage matrix metalloproteinase-12 dampens inflammation and neutrophil influx in arthritis**
Resolution of inflammation reduces pathological tissue destruction and restores tissue homeostasis. Here, we used a proteomic protease substrate discovery approach, terminal amine isotopic labeling of substrates (TAILS), to analyze the role of the macrophage-specific matrix metalloproteinase-12 (MMP12) in inflammation. In murine peritonitis, MMP12 inactivates antithrombin and activates prothrombin, prolonging the activated partial thromboplastin time. Furthermore, MMP12 inactivates complement C3 to reduce complement activation and inactivates the chemoattractant anaphylatoxins C3a and C5a, whereas iC3b and C3b opsonin cleavage increases phagocytosis. Loss of these anti-inflammatory activities in collagen-induced arthritis in Mmp12−/− mice leads to unresolved synovitis and extensive articular inflammation. Deep articular cartilage loss is associated with massive neutrophil infiltration and abnormal DNA neutrophil extracellular traps (NETs). The NETs are rich in fibrin and extracellular actin, which TAILS identified as MMP12 substrates. Thus, macrophage MMP12 in arthritis has multiple protective roles in countering neutrophil infiltration, clearing NETs, and dampening inflammatory pathways to prepare for the resolution of inflammation.

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Time-resolved analysis of the matrix metalloproteinase 10 substrate degradome

Proteolysis is an irreversible post-translational modification that affects intra- and intercellular communication by modulating the activity of bioactive mediators. Key to understanding protease function is the system-wide identification of cleavage events and their dynamics in physiological contexts. Despite recent advances in mass spectrometry-based proteomics for high-throughput substrate screening, current approaches suffer from high false positive rates and only capture single states of protease activity. Here, we present a workflow based on multiplexed terminal amine isotopic labeling of substrates for timeresolved substrate degradomics in complex proteomes. This approach significantly enhances confidence in substrate identification and categorizes cleavage events by specificity and structural accessibility of the cleavage site. We demonstrate concomitant quantification of cleavage site spanning peptides and neo-N and/or neo-C termini to estimate relative ratios of noncleaved and cleaved forms of substrate proteins. By applying this strategy to dissect the matrix metalloproteinase 10 (MMP10) substrate degradome in fibroblast secretomes, we identified the extracellular matrix protein ADAMTS-like protein 1 (ADAMTSL1) as a direct MMP10 substrate and revealed MMP10-dependent ectodomain shedding of platelet-derived growth factor receptor alpha (PDGFRα) as well as sequential processing of type I collagen. The data have been deposited to the ProteomeXchange Consortium with identifier PXD000503.

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The substrate degradome of meprin metalloproteases reveals an unexpected proteolytic link between meprin β and ADAM10

The in vivo roles of meprin metalloproteases in pathophysiological conditions remain elusive. Substrates define protease roles. Therefore, to identify natural substrates for human meprin α and β we employed TAILS (terminal amine isotopic labeling of substrates), a proteomics approach that enriches for N-terminal peptides of proteins and cleavage fragments. Of the 151 new extracellular substrates we identified, it was notable that ADAM10 (a disintegrin and metalloprotease domain-containing protein 10)-the constitutive α-secretase-is activated by meprin β through cleavage of the propeptide. To validate this cleavage event, we expressed recombinant proADAM10 and after preincubation with meprin β, this resulted in significantly elevated ADAM10 activity. Cellular expression in murine primary fibroblasts confirmed activation. Other novel substrates including extracellular matrix proteins, growth factors and inhibitors were validated by western analyses and enzyme activity assays with Edman sequencing confirming the exact cleavage sites identified by TAILS. Cleavages in vivo were confirmed by comparing wild-type and meprin-/- mice. Our finding of cystatin C, elafin and fetuin-A as substrates and natural inhibitors for meprins reveal new mechanisms in the regulation of protease activity important for understanding pathophysiological processes.

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CLIPPER: An add-on to the Trans-Proteomic Pipeline for the automated analysis of TAILS N-terminomics data

Data analysis in proteomics is complex and with the extra challenges involved in the interpretation of data from N-terminomics experiments, this can be daunting. Therefore, we have devised a rational pipeline of steps to approach N-terminomics data analysis in a statistically-based and valid manner. We have automated these steps in CLIPPER, an add-on to the Trans-Proteomic Pipeline (TPP). Applying CLIPPER to the analysis of N-terminomics data generated by terminal amine isotopic labeling of substrates (TAILS) enables high confidence peptide to protein assignment, protein N-terminal characterization and annotation, and for protease analysis readily allows protease substrate discovery with high confidence.
Identification of UV-protective activators of nuclear factor erythroid-derived 2-related factor 2 (Nrf2) by combining a chemical library screen with computer-based virtual screening

Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is a master regulator of cellular antioxidant defense systems, and activation of this transcription factor is a promising strategy for protection of skin and other organs from environmental insults. To identify efficient Nrf2 activators in keratinocytes, we combined a chemical library screen with computer-based virtual screening. Among 14 novel Nrf2 activators, the most potent compound, a nitrophenyl derivative of 2-chloro-5-nitro-N-phenyl-benzamide, was characterized with regard to its molecular mechanism of action. This compound induced the expression of cytoprotective genes in keratinocytes isolated from wild-type but not from Nrf2-deficient mice. Most importantly, it showed low toxicity and protected primary human keratinocytes from UVB-induced cell death. Therefore, it represents a potential lead compound for the development of drugs for skin protection under stress conditions. Our study demonstrates that chemical library screening combined with advanced computational similarity searching is a powerful strategy for identification of bioactive compounds, and it points toward an innovative therapeutic approach against UVB-induced skin damage.

Characterization of the prime and non-prime active site specificities of proteases by proteome-derived peptide libraries and tandem mass spectrometry

To link cleaved substrates in complex systems with a specific protease, the protease active site specificity is required. Proteomic identification of cleavage sites (PICS) simultaneously determines both the prime-and non-prime-side specificities of individual proteases through identification of hundreds of individual cleavage sequences from biologically relevant, proteome-derived peptide libraries. PICS also identifies subsite cooperativity. To generate PICS peptide libraries, cellular proteomes are digested with a specific protease such as trypsin. Following protease inactivation, primary amines are protected. After incubation with a test protease, each prime-side cleavage fragment has a free newly formed N-terminus, which is biotinylated for affinity isolation and identification by liquid chromatography-tandem mass spectrometry. The corresponding non-prime sequences are derived bioinformatically. The step-by-step protocol also presents a web service for PICS data analysis, as well as introducing and validating PICS peptide libraries made from Escherichia coli.
Factor Xa subsite mapping by proteome-derived peptide libraries improved using WebPICS, a resource for proteomic identification of cleavage sites

Proteomic identification of protease cleavage site specificity (PICS) is a recent proteomic approach for the easy mapping of protease subsite preferences that determines both the prime- and non-prime side specificity concurrently. Here we greatly facilitate user access by providing an automated and simple web-based data-analysis resource termed WebPics (http://clipserve.clip.ubc.ca/ pics/). We demonstrate the utility of WebPics analysis of PICS data by determining the substrate specificity of factor Xa from P6-P6', an important blood coagulation protease that proteolytically generates thrombin from prothrombin. PICS confirms existing data on non-prime site specificity and refines our knowledge of factor Xa prime-site selectivity.

Identifying and quantifying proteolytic events and the natural N terminome by terminal amine isotopic labeling of substrates

Analysis of the sequence and nature of protein N termini has many applications. Defining the termini of proteins for proteome annotation in the Human Proteome Project is of increasing importance. Terminomics analysis of protease cleavage sites in degradomics for substrate discovery is a key new application. Here we describe the step-by-step procedures for performing terminal amine isotopic labeling of substrates (TAILS), a 2- to 3-d (depending on method of labeling) high-throughput method to identify and distinguish protease-generated neo-N termini from mature protein N termini with all natural modifications with high confidence. TAILS uses negative selection to enrich for all N-terminal peptides and uses primary amine labeling-based quantification as the discriminating factor. Labeling is versatile and suited to many applications, including biochemical and cell culture analyses in vitro; in vivo analyses using tissue samples from animal and human sources can also be readily performed. At the protein level, N-terminal and lysine amines are blocked
by dimethylation (formaldehyde/sodium cyanoborohydride) and isotopically labeled by incorporating heavy and light dimethylation reagents or stable isotope labeling with amino acids in cell culture labels. Alternatively, easy multiplex sample analysis can be achieved using amine blocking and labeling with isobaric tags for relative and absolute quantification, also known as iTRAQ. After tryptic digestion, N-terminal peptide separation is achieved using a high-molecular-weight dendritic polyglycerol aldehyde polymer that binds internal tryptic and C-terminal peptides that now have N-terminal alpha amines. The unbound naturally blocked (acetylation, cyclization, methylation and so on) or labeled mature N-terminal and neo-N-terminal peptides are recovered by ultrafiltration and analyzed by tandem mass spectrometry (MS/MS). Hierarchical substrate winnowing discriminates substrates from the background proteolysis products and non-cleaved proteins by peptide isotope quantification and bioinformatics search criteria.

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Corresponding author: Overall, C. M.
Contributors: Kleifeld, O., Doucet, A., Prudova, A., Auf Dem Keller, U., Gioia, M., Kizhakkedathu, J. N., Overall, C. M.
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**Metalloprotease meprin β generates nontoxic N-terminal amyloid precursor protein fragments in vivo**

Identification of physiologically relevant substrates is still the most challenging part in protease research for understanding the biological activity of these enzymes. The zinc-dependent metalloprotease meprin β is known to be expressed in many tissues with functions in health and disease. Here, we demonstrate unique interactions between meprin β and the amyloid precursor protein (APP). Although APP is intensively studied as a ubiquitously expressed cell surface protein, which is involved in Alzheimer disease, its precise physiological role and relevance remain elusive. Based on a novel proteomics technique termed terminal amine isotopic labeling of substrates (TAILS), APP was identified as a substrate for meprin β. Processing of APP by meprin β was subsequently validated using in vitro and in vivo approaches. N-terminal APP fragments of about 11 and 20 kDa were found in human and mouse brain lysates but not in meprin β -/- mouse brain lysates. Although these APP fragments were in the range of those responsible for caspase-induced neurodegeneration, we did not detect cytotoxicity to primary neurons treated by these fragments. Our data demonstrate that meprin β is a physiologically relevant enzyme in APP processing.

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Corresponding author: Pietrzik, C. U.
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Nematotoxicity of Marasmius oreades Agglutinin (MOA) depends on glycolipid binding and cysteine protease activity

Fruiting body lectins have been proposed to act as effector proteins in the defense of fungi against parasites and predators. The Marasmius oreades agglutinin (MOA) is a Galα1,3Gal/GalNAc-specific lectin from the fairy ring mushroom that consists of an N-terminal ricin B-type lectin domain and a C-terminal dimerization domain. The latter domain shows structural similarity to catalytically active proteins, suggesting that, in addition to its carbohydrate-binding activity, MOA has an enzymatic function. Here, we demonstrate toxicity of MOA toward the model nematode Caenorhabditis elegans. This toxicity depends on binding of MOA to glycosphingolipids of the worm via its lectin domain. We show further that MOA has cysteine protease activity and demonstrate a critical role of this catalytic function in MOA-mediated nematotoxicity. The proteolytic activity of MOA was dependent on high Ca$^{2+}$ concentrations and favored by slightly alkaline pH, suggesting that these conditions trigger activation of the toxin at the target location. Our results suggest that MOA is a fungal toxin with intriguing similarities to bacterial binary toxins and has a protective function against fungivorous soil nematodes.
simultaneously profiles the specificity of prime and nonprime positions and directly determines scissile peptide bonds of up to hundreds of cleavage site sequences in a single experiment. This wealth of sequence specificity information also allows for the investigation of subsite cooperativity. Herein we describe a simplified procedure to produce PICS peptide libraries, the methods to perform a PICS assay, and a new method of data analysis.

**Proteomic analyses reveal an acidic prime side specificity for the astacin metalloprotease family reflected by physiological substrates**

Astacins are secreted and membrane-bound metalloproteases with clear associations to many important pathological and physiological processes. Yet with only a few substrates described their biological roles are enigmatic. Moreover, the lack of knowledge of astacin cleavage site specificities hampers assay and drug development. Using PICS (proteomic identification of protease cleavage site specificity) and TAILS (terminal amine isotopic labeling of substrates) degradomics approaches, >3000 cleavage sites were proteomically identified for five different astacins. Such broad coverage enables family-wide determination of specificities N- and C-terminal to the scissile peptide bond. Remarkably, meprin α, meprin β, and LAST-MAM proteases exhibit a strong preference for aspartate in the peptide (P)1′ position because of a conserved positively charged residue in the active cleft subsite (S)1′. This unparalleled specificity has not been found for other families of extracellular proteases. Interestingly, cleavage specificity is also strongly influenced by proline in P2′ or P3′ leading to a rare example of subsite cooperativity. This specificity characterizes the astacins as unique contributors to extracellular proteolysis that is corroborated by known cleavage sites in procollagen I+III, VEGF (vascular endothelial growth factor)-A, IL (interleukin)-1β, and pro-kallikrein 7. Indeed, cleavage sites in VEGF-A and pro-kallikrein 7 identified by terminal amine isotopic labeling of substrates matched those reported by Edman degradation. Moreover, the novel substrate FGF-19 was validated biochemically and shown to exhibit altered biological activity after meprin processing.

**General information**

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Contributors: Schilling, O., Keller, U. A. D., Overall, C. M.
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**Proteomic analyses reveal an acidic prime side specificity for the astacin metalloprotease family reflected by physiological substrates**

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Corresponding author: Becker-Pauly, C.
Contributors: Becker-Pauly, C., Barré, O., Schilling, O., Auf Dem Keller, U., Ohler, A., Broder, C., Schütte, A., Kappelhoff, R., Stöcker, W., Overall, C. M.
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- Web of Science (2011): Impact factor 7.398
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- Web of Science (2011): Indexed yes
Targeting Tumor Hypoxia: Suppression of Breast Tumor Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors

Carbonic anhydrase IX (CAIX) is a hypoxia and HIF-1–inducible protein that regulates intra- and extracellular pH under hypoxic conditions and promotes tumor cell survival and invasion in hypoxic microenvironments. Interrogation of 3,630 human breast cancers provided definitive evidence of CAIX as an independent poor prognostic biomarker for distant metastases and survival. shRNA-mediated depletion of CAIX expression in 4T1 mouse metastatic breast cancer cells capable of inducing CAIX in hypoxia resulted in regression of orthotopic mammary tumors and inhibition of spontaneous lung metastasis formation. Stable depletion of CAIX in MDA-MB-231 human breast cancer xenografts also resulted in attenuation of primary tumor growth. CAIX depletion in the 4T1 cells led to caspase-independent cell death and reversal of extracellular acidosis under hypoxic conditions in vitro. Treatment of mice harboring CAIX-positive 4T1 mammary tumors with novel CAIX-specific small molecule inhibitors that mimicked the effects of CAIX depletion in vitro resulted in significant inhibitory effects on CAIX-negative tumors. Similar inhibitory effects on primary tumor growth were observed in mice harboring orthotopic tumors comprised of lung metastatic MDA-MB-231 LM2-4Luc+ cells. Our findings show that CAIX is vital for growth and metastasis of hypoxic breast tumors and is a specific, targetable biomarker for breast cancer metastasis. Cancer Res; 71(9); 3364–76. ©2011 AACR.

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Wound degradomics – current status and future perspectives
Proteases are pivotal modulators of extracellular matrix components and bioactive proteins at all phases of cutaneous wound healing and thereby essentially contribute to the successful reestablishment of skin integrity upon injury. As a consequence, disturbance of proteolytic activity at the wound site is a major factor in the pathology of chronic wounds. A large body of data acquired in many years of research provide a good understanding of how individual proteases may influence the repair process. The next challenge will be to integrate these findings and to elucidate the complex interactions of proteolytic enzymes, their inhibitors and substrates on a system-wide level. Here, we present novel approaches that might help to achieve this ambitious goal in cutaneous wound healing research.

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Wound degradomics – current status and future perspectives
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Analysis of the Degradome with the CLIP-CHIP™ Microarray

The degradome microarray – CLIP-CHIP™ – is a dedicated and focused array that allows the analysis of all proteases, non-proteolytic homologs, and protease inhibitor gene transcripts in the human and murine genomes at the mRNA transcript level.

Based on unique 70-mer oligonucleotides, designed to match parts of the sequence of known or predicted protease and inhibitor mRNAs in both species and printed on a glass-matrix surface, the CLIP-CHIP™ microarray can be used to analyze differentially expressed protease and inhibitor gene products and give expression profiles for any analyzed sample.

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Contributors: Kappelhoff, R., auf dem Keller, U., Overall, C. M.
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A statistics-based platform for quantitative N-terminome analysis and identification of protease cleavage products

Terminal amine isotopic labeling of substrates (TAILS), our recently introduced platform for quantitative N-terminome analysis, enables wide dynamic range identification of original mature protein N-termini and protease cleavage products. Modifying TAILS by use of isobaric tag for relative and absolute quantification (iTRAQ)-like labels for quantification together with a robust statistical classifier derived from experimental protease cleavage data, we report reliable and statistically valid identification of proteolytic events in complex biological systems in MS2 mode. The statistical classifier is supported by a novel parameter evaluating ion intensity-dependent quantification confidences of single peptide quantifications, the quantification confidence factor (QCF). Furthermore, the isoform assignment score (IAS) is introduced, a new scoring system for the evaluation of single peptide-to-protein assignments based on high confidence protein identifications in the same sample prior to negative selection enrichment of N-terminal peptides. By these approaches, we identified and validated, in addition to known substrates, low abundance novel bioactive MMP-2 targets including the plasminogen receptor S100A10 (p11) and the proinflammatory cytokine proEMAP/p43 that were previously undescribed.

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Organisations: Swiss Federal Institute of Technology Zurich, University of British Columbia
Contributors: Auf Dem Keller, U., Prudova, A., Gioia, M., Butler, G. S., Overall, C. M.
Isotopic labeling of terminal amines in complex samples identifies protein N-termini and protease cleavage products

Effective proteome-wide strategies that distinguish the N-termini of proteins from the N-termini of their protease cleavage products would accelerate identification of the substrates of proteases with broad or unknown specificity. Our approach, named terminal amine isotopic labeling of substrates (TAILS), addresses this challenge by using dendritic polyglycerol aldehyde polymers that remove tryptic and C-terminal peptides. We analyze unbound naturally acetylated, cyclized or labeled N-termini from proteins and their protease cleavage products by tandem mass spectrometry, and use peptide isotope quantification to discriminate between the substrates of the protease of interest and the products of background proteolysis. We identify 731 acetylated and 132 cyclized N-termini, and 288 matrix metalloproteinase (MMP)-2 cleavage sites in mouse fibroblast secretomes. We further demonstrate the potential of our strategy to link proteases with defined biological pathways in complex samples by analyzing mouse inflammatory bronchoalveolar fluid and showing that expression of the poorly defined breast cancer protease MMP-11 in MCF-7 human breast cancer cells cleaves both endoplasmin and the immunomodulator and apoptosis inducer galectin-1.

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Multiplex N-terminome Analysis of MMP-2 and MMP-9 Substrate Degradomes by TTRAQ-TAILS Quantitative Proteomics

Proteolysis is a major protein posttranslational modification that, by altering protein structure, affects protein function and, by truncating the protein sequence, alters peptide signatures of proteins analyzed by proteomics. To identify such modified
and shortened protease-generated neo-N-termini on a proteome-wide basis, we developed a whole protein isobaric tag for relative and absolute quantitation (iTRAQ) labeling method that simultaneously labels and blocks all primary amines including protein N-termini and lysine side chains. Blocking lysines limits trypsin cleavage to arginine, which effectively elongates the proteolytically truncated peptides for improved MS/MS analysis and peptide identification. Incorporating iTRAQ whole protein labeling with terminal amine isotopic labeling of substrates (iTRAQ-TAILS) to enrich the N-termine by negative selection of the blocked mature original N-termini and neo-N-termini has many advantages. It enables simultaneous characterization of the natural N-termini of proteins, their N-terminal modifications, and proteolysis product and cleavage site identification. Furthermore, iTRAQ-TAILS also enables multiplex N-terminomics analysis of up to eight samples and allows for quantification in MS2 mode, thus preventing an increase in spectral complexity and extending proteome coverage by signal amplification of low abundance proteins. We compared the substrate degradomes of two closely related matrix metalloproteinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), in fibroblast secreted proteins. Among 3,152 unique N-terminal peptides identified corresponding to 1,054 proteins, we detected 201 cleavage products for MMP-2 and unexpectedly only 19 for the homologous MMP-9 under identical conditions. Novel substrates identified and biochemically validated include insulin-like growth factor binding protein-4, complement C1r component A, galectin-1, dickkopf-related protein-3, and thrombospondin-2. Hence, N-terminomics analyses using iTRAQ-TAILS links gelatinases with new mechanisms of action in angiogenesis and reveals unpredicted restrictions in substrate repertoires for these two very similar proteases.

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Novel matrix metalloproteinase inhibitor [18F]marimastat- arytrifluoroborate as a probe for in vivo positron emission tomography imaging in cancer
Matrix metalloproteinases (MMP), strongly associated pathogenic markers of cancer, have undergone extensive drug development programs. Marimastat, a noncovalent MMP inhibitor, was conjugated with FITC to label cellular metalloproteinase cancer targets in MDA-MB-231 cells in vitro. Punctate localization of active transmembrane MMP14 was observed. For molecular-targeted positron emission tomography imaging of syngeneic 67NR murine mammary carcinoma in vivo, marimastat was [18F]labeled using a shelf-stable arybongenic ester conjugate as a captor for aqueous [18F]fluoride in a novel, rapid one-step reaction at ambient temperature. [18F]Marimastat- arytrifluoroborate localized to the tumors, with labeling being blocked in control animals first loaded with >10-fold excess unlabeled marimastat. The labeled drug cleared primarily via the hepatobiliary and gastrointestinal tract, with multiple animals imaged in independent experiments, confirming the ease of this new labeling strategy.

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Nrf2 A central regulator of UV protection in the epidermis

Ultraviolet (UV) B irradiation can severely damage the skin and even induce tumorigenesis. It exerts its effects by direct DNA modification and by formation of reactive oxygen species (ROS). We developed a strategy to genetically activate target gene expression of the transcription factor NF-E2-related factor 2 (Nrf2) in keratinocytes in vivo based on expression of a constitutively active Nrf2 mutant. Activation of Nrf2 target genes strongly reduced UVB cytotoxicity through enhancement of ROS detoxification. Remarkably, the protective effect was extended to neighboring cells. Using different combinations of genetically modified mice, we demonstrate that Nrf2 activates the production, recycling, and release of glutathione and cysteine by suprabasal keratinocytes, resulting in protection of basal cells in a paracrine, glutathione/cysteine-dependent manner. Most importantly, we found that endogenous Nrf2 controls selective protection of suprabasal keratinocytes from UVB-induced apoptosis through activation of cytoprotective genes. This finding explains the preferential UVB-induced apoptosis of basal cells, which is important for elimination of mutated stem cells as well as for preservation of skin integrity. Taken together, our results identify Nrf2 as a key regulator in the UV response of the skin.
Proteomic techniques and activity-based probes for the system-wide study of proteolysis

Proteolysis constitutes a major post-translational modification but specificity and substrate selectivity of numerous proteases have remained elusive. In this review, we highlight how advanced techniques in the areas of proteomics and activity-based probes can be used to investigate i) protease active site specificity; ii) protease in vivo substrates; iii) protease contribution to proteome homeostasis and composition; and iv) detection and localization of active proteases. Peptide libraries together with genetical or biochemical selection have traditionally been used for active site profiling of proteases. These are now complemented by proteome-derived peptide libraries that simultaneously determine prime and non-prime specificity and characterize subsite cooperativity. Cell-contextual discovery of protease substrates is rendered possible by techniques that isolate and quantitate protein termini. Here, a novel approach termed Terminal Amine Isotopic Labeling of Substrates (TAILS) provides an integrated platform for substrate discovery and appropriate statistical evaluation of terminal peptide identification and quantification. Proteolytically generated carboxy-termini can now also be analyzed on a proteome-wide level. Proteolytic regulation of proteome composition is monitored by quantitative proteomic approaches employing stable isotope coding or label free quantification. Activity-based probes specifically recognize active proteases. In proteomic screens, they can be used to detect and quantitate proteolytic activity while their application in cellular histology allows to locate proteolytic activity in situ. Activity-based probes - especially in conjunction with positron emission tomography - are also promising tools to monitor proteolytic activities on an organism-wide basis with a focus on in vivo tumor imaging. Together, this array of methodological possibilities enables unveiling physiological protease substrate repertoires and defining protease function in the cellular- and organism-wide context.
Epithelial-Mesenchymal Transition (EMT) Is Not Sufficient for Spontaneous Murine Breast Cancer Metastasis

Epithelial-mesenchymal transition (EMT) has been linked to metastatic propensity. The 4T1 tumor is a clinically relevant model of spontaneous breast cancer metastasis. Here we characterize 4T1-derived cell lines for EMT, in vitro invasiveness and in vivo metastatic ability. Contrary to expectations, 67NR cells, which form primary tumors but fail to metastasize, express vimentin and N-cadherin, but not E-cadherin. 4T1 cells express E-cadherin and ZO-1, but are migratory, invasive, and metastasize to multiple sites. 66c14 cells form lung metastases and display a mixed phenotype, but are not as migratory or invasive as 67NR cells. These findings demonstrate that the metastatic ability of breast cancer cells does not strictly correlate with genotypic and phenotypic properties of EMT per se, and suggest that other processes may govern metastatic capability. Gene expression analysis of primary tumors did not identify differences in EMT markers, but did reveal candidate genes that may influence metastatic ability. Developmental Dynamics 237:2755-2768, 2008. (C) 2008 Wiley-Liss, Inc.

Identification of protease substrates by mass spectrometry approaches-2

Proteolysis is a major posttranslational modification of proteins with critical functional consequences to the protein, cell, and organism. The most effective way to monitor proteolytic events is to analyze the proteins directly. This chapter summarizes advantages and limitations of different mass spectrometry-based approaches for detection of proteolysis products. In general, liquid chromatography separation-based proteomics approaches are superior to 2D gel-based techniques and, in turn, quantitative proteomics have a significant advantage over label-free methods. Isotopic labeling of samples helps to identify substrates but fails to detect the exact cleavage site. Techniques that enrich for peptides containing the N-terminus of each protein provide a more relevant context for protease substrate discovery -they focus on the analysis of the neo-N-termini resulting from proteolysis. These techniques identify not only the substrates but also the prime side of the cleavage sites with a potential to extract further information of the protease sequence site specificity, thus setting the gold standard for the future of the degradomics field.
Impaired liver regeneration in Nrf2 knockout mice: Role of ROS-mediated insulin/IGF-1 resistance

The liver is frequently challenged by surgery-induced metabolic overload, viruses or toxins, which induce the formation of reactive oxygen species. To determine the effect of oxidative stress on liver regeneration and to identify the underlying signaling pathways, we studied liver repair in mice lacking the Nrf2 transcription factor. In these animals, expression of several cytoprotective enzymes was reduced in hepatocytes, resulting in oxidative stress. After partial hepatectomy, liver regeneration was significantly delayed. Using in vitro and in vivo studies, we identified oxidative stress-mediated insulin/insulin-like growth factor resistance as an underlying mechanism. This deficiency impaired the activation of p38 mitogen-activated kinase, Akt kinase and downstream targets after hepatectomy, resulting in enhanced death and delayed proliferation of hepatocytes. Our results reveal novel roles of Nrf2 in the regulation of growth factor signaling and in tissue repair. In addition, they provide new insight into the mechanisms underlying oxidative stress-induced defects in liver regeneration. These findings may provide the basis for the development of new strategies to improve regeneration in patients with acute or chronic liver damage.

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Toward \[^{18}F\]-labeled aryltrifluoroborate radiotracers: In vivo positron emission tomography imaging of stable aryltrifluoroborate clearance in mice

The use of a boronic ester as a captor of aqueous \[^{18}F\]-fluoride has been previously suggested as a means of labeling biomolecules in one step for positron emission tomography (PET) imaging. For this approach to be seriously considered, the \[^{18}F\]-labeled trifluoroborate should be humorally stable such that it neither leaches free \[^{18}F\]-fluoride to the bone nor accumulates therein. Herein, we have synthesized a biotinylated boronic ester that is converted to the corresponding trifluoroborate salt in the presence of aqueous \[^{18}F\]-fluoride. In keeping with its in vitro aqueous kinetic stability at pH 7.5, the trifluoroborate appears to clear in vivo quite rapidly to the bladder as the stable trifluoroborate salt with no detectable leaching of free \[^{18}F\]-fluoride to the bone. When this labeled biotin is preincubated with avidin, the pharmacokinetic clearance of the resulting complex is visibly altered. This work validates initial claims that boronic esters are potentially useful as readily labeled precursors to \[^{18}F\]-PET reagents.

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Electrophilic chemicals but not UV irradiation or reactive oxygen species activate Nrf2 in keratinocytes in vitro and in vivo

The NF-E2-related factor 2 (Nrf2) transcription factor is a potent inducer of cytoprotective genes, which encode - among others - enzymes that detoxify reactive oxygen species (ROS). As we demonstrated a crucial role of Nrf2 in the prevention of skin carcinogenesis, it is of interest to identify Nrf2-activating factors in keratinocytes. For this purpose, keratinocytes from mice transgenic for an Nrf2-responsive reporter gene were analyzed. Electrophilic compounds activated the reporter in keratinocytes, and induced nuclear translocation of Nrf2 and the expression of known Nrf2 target genes. This is biologically relevant, as we show that Nrf2-mediated gene expression protects keratinocytes from the toxicity of these substances. By contrast, hydrogen peroxide, glucose oxidase, UVA, and UVB irradiation had no effect, although these treatments strongly increased the levels of intracellular ROS. To verify these results in vivo, transgenic reporter mice with and without functional Nrf2 alleles were topically treated with electrophilic chemicals or irradiated with UVB. Electrophiles but not UVB activated the reporter in an Nrf2-dependent manner. These results provide the basis for the identification of novel Nrf2 activators in keratinocytes with therapeutic potential for skin tumor prevention.

Protease research in the era of systems biology

Proteases are specific modulators of signaling molecules and their underlying pathways in addition to their degradative roles. However, proteases do not act alone, but form cascades, circuits and networks that all dynamically interconnect to form the protease web, which defines the proteolytic potential of a cell or tissue in a defined condition. To describe the protease web and its net activity several novel high-throughput proteomic techniques, in the field termed degradomics, have been developed. Emerging systems biology methods to evaluate the expression, activity and substrate discovery of proteases are presented. Understanding the protease web and its perturbations in pathology will help to develop new therapeutics for the treatment of diseases, such as cancer, arthritis and chronic obstructive pulmonary diseases.
Roles and mechanisms of action of the Nrf2 transcription factor in skin morphogenesis, wound repair and skin cancer

Nrf transcription factors in keratinocytes are essential for skin tumor prevention but not for wound healing

The Nrf2 transcription factor is a key player in the cellular stress response through its regulation of cytoprotective genes. In this study we determined the role of Nrf2-mediated gene expression in keratinocytes for skin development, wound repair, and skin carcinogenesis. To overcome compensation by the related Nrf1 and Nrf3 proteins, we expressed a dominant-negative Nrf2 mutant (dnNrf2) in the epidermis of transgenic mice. The functionality of the transgene product was verified in vivo using mice doubly transgenic for dnNrf2 and an Nrf2-responsive reporter gene. Surprisingly, no abnormalities of the epidermis were observed in dnNrf2-transgenic mice, and even full-thickness skin wounds healed normally. However, the onset, incidence, and multiplicity of chemically induced skin papillomas were strikingly enhanced, whereas the progression to squamous cell carcinomas was unaltered. We provide evidence that the enhanced tumorigenesis results from reduced basal expression of cytoprotective Nrf target genes, leading to accumulation of oxidative damage and reduced carcinogen detoxification. Our results reveal a crucial role of Nrf-mediated gene expression in keratinocytes in the prevention of skin tumors and suggest that activation of Nrf2 in keratinocytes is a promising strategy to prevent carcinogenesis of this highly exposed organ.
Reactive oxygen species and their detoxification in healing skin wounds

Injury to the skin initiates a cascade of events, which finally lead to at least partial reconstruction of the wounded tissue. The wound-healing process has been well described at the histological level, but the underlying molecular mechanisms are still poorly defined. To gain insight into these mechanisms we searched for genes, which are regulated by skin injury. Interestingly, some of the genes that we identified encode cytoprotective proteins, in particular enzymes, which detoxify reactive oxygen species (ROS). Since ROS are produced in high amounts at the wound site as a defense against invading bacteria, the expression of these genes is most likely important for the protection of cells against these toxic molecules. In this review, we summarize the results on the expression of cytoprotective genes in wounded skin, and we discuss their possible roles in the wound-healing process.

Fibroblast growth factors in epithelial repair and cytoprotection

Growth factors are polypeptides that stimulate the division of certain cell types at low concentrations. Fibroblast growth factor (FGF) 7 (FGF-7) and its homologue FGF-10 act specifically on various types of epithelial cells including keratinocytes of the skin, intestinal epithelial cells and hepatocytes. In addition, FGF-7 and FGF-10 have been shown to be more than growth factors: they can protect epithelial cells from damaging effects induced, for example, by radiation and oxidative stress. Therefore, they are currently in clinical trials for the treatment of oral mucositis, a severe side-effect of cancer therapy characterized by painful inflammation and ulceration of the oral epithelium. To gain insight into the mechanisms of FGF-7/FGF-10 action in epithelial cells, we searched for genes that are regulated by these growth factors.
Indeed, we identified genes that help us to explain the mechanisms that underlie the effects of FGF-7. Most interestingly, several genes were identified that are likely to mediate the cytoprotective effect of FGF-7 for epithelial cells in vitro and possibly also in injured and diseased tissues in vivo.

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Meeting report: Growth factors in development, repair and disease

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Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound

Keratinocyte growth factor (KGF) is a potent mitogen for epithelial cells, and it promotes survival of these cells under stress conditions. In a search for KGF-regulated genes in keratinocytes, we identified the gene encoding the transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 is a key player in the cellular stress response. This might be of particular importance during wound healing, where large amounts of reactive oxygen species are produced as a defense against invading bacteria. Therefore, we studied the wound repair process in Nrf2 knockout mice. Interestingly, the expression of various key players involved in wound healing was significantly reduced in early wounds of the Nrf2 knockout animals, and the late phase of repair was characterized by prolonged inflammation. However, these differences in gene expression were not reflected by obvious histological abnormalities. The normal healing rate appears to be at least partially due to an up-regulation of the related transcription factor Nrf3, which was also identified as a target of KGF and which was coexpressed with Nrf2 in the healing skin wound. Taken together, our results reveal novel roles of the KGF-regulated transcription factors Nrf2 and possibly Nrf3 in the control of gene expression and inflammation during cutaneous wound
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Projects:

Molecular Insight of Alginate Degradation by Human Gut Microbiota and Host Interplay
Renne, M. E., PhD Student, Department of Systems Biology
Svensson, B., Main Supervisor
Pedersen, S. B., Supervisor
auf dem Keller, U., Supervisor
01/09/2019 → 31/08/2022
Project: PhD

Structural and functional studies of MARCH5
Merklinger, L., PhD Student, Department of Systems Biology
Morth, J. P., Main Supervisor
Pedersen, S. B., Supervisor
auf dem Keller, U., Supervisor
01/01/2019 → 31/12/2021
Project: PhD

Wound exudate degradomics in normal and impaired healing
Kalogeropoulos, K., PhD Student, Department of Systems Biology
Laustsen, A. H., Supervisor
Bundgaard, L., Supervisor
01/04/2019 → 31/03/2022
Project: PhD

pH-sensing Monoclonal antibodies against snake toxins
Jensen, L. L., PhD Student, Department of Systems Biology
Laustsen, A. H., Main Supervisor
auf dem Keller, U., Supervisor
01/03/2019 → 28/02/2022
Project: PhD

Immune stealthing of bio-therapeutics
Lie-Andersen, O., PhD Student, Department of Systems Biology

repair.
Structural and cellular studies of MARCH5 and larger complexes
Müller, E., PhD Student, Department of Systems Biology
Morth, J. P., Main Supervisor
auf dem Keller, U., Supervisor
01/01/2019 → 31/12/2021
Project: PhD

Functional modification of matrix metalloproteinase (MMP) 9 activity by glycosylation
Madzharova, E., PhD Student, Department of Systems Biology
auf dem Keller, U., Main Supervisor
Brix, S., Supervisor
01/12/2017 → 30/11/2020
Award relations: Functional modification of matrix metalloproteinase (MMP) 9 activity by glycosylation
Project: PhD

Interconnected Activities and Functions of Matrix Metalloproteinases at the Wound Edge
Savickas, S., PhD Student, Department of Systems Biology
Svensson, B., Main Supervisor
01/09/2017 → 31/08/2020
Award relations: Interconnected Activities and Functions of Matrix Metalloproteinases at the Wound Edge
Project: PhD

Characterization of novel non-invasive biomarkers for extracellular matrix remodeling in fibrosis and discovery of new targets for biomarker development
Nielsen, S. H., PhD Student, Department of Systems Biology
Pederssen, S. B., Main Supervisor
Genovese, F., Supervisor
Leeming, D. J., Supervisor
01/03/2015 → 20/06/2018
Award relations: Characterization of novel non-invasive biomarkers for extracellular matrix remodeling in fibrosis and discovery of new targets for biomarker development
Project: PhD

Keratin2Protein: Novel approach to protein recovery from slaughterhouse waste through microbial conversion
Espersen, R., PhD Student, Department of Systems Biology
Svensson, B., Main Supervisor
Abou Hachem, M., Supervisor
Hägglund, P., Supervisor
01/12/2014 → 20/06/2018
Award relations: Keratin2Protein: Novel approach to protein recovery from slaughterhouse waste through microbial conversion
Project: PhD
Novel Biomarkers of changes in muscle mass or muscle pathology
Arvanitidis, A., PhD Student, Department of Systems Biology
Svensson, B., Main Supervisor
Hägglund, P., Supervisor
auf dem Keller, U., Examiner
Kjær, M., Examiner
Sorsa, T. A., Examiner
Industrial PhD
01/01/2014 → 24/01/2018
Award relations: Novel Biomarkers of changes in muscle mass or muscle pathology
Project: PhD