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Review

Positional proteomics in the era of the human proteome project on the doorstep of precision medicine

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ABSTRACT

Proteolytic processing is a pervasive and irreversible post-translational modification that expands the protein universe by generating new proteoforms (protein isoforms). Unlike signal peptide or prodomain removal, protease-generated proteoforms can rarely be predicted from gene sequences. Positional proteomic techniques that enrich for N- or C-terminal peptides from proteomes are indispensable for a comprehensive understanding of a protein's function in biological environments since protease cleavage frequently results in altered protein activity and localization. Proteases often process other proteases and protease inhibitors which perturbs proteolytic networks and potentiates the initial cleavage event to affect other molecular networks and cellular processes in physiological and pathological conditions. This review is aimed at researchers with a keen interest in state of the art systems level positional proteomic approaches that: (i) enable the study of complex protease–protease, protease-inhibitor and protease-substrate crosstalk and networks; (ii) allow the identification of proteolytic signatures as candidate disease biomarkers; and (iii) are expected to fill the Human Proteome Project missing proteins gap. We predict that these methodologies will be an integral part of emerging precision medicine initiatives that aim to customize healthcare, converting reactive medicine into a personalized and proactive approach, improving clinical care and maximizing patient health and wellbeing, while decreasing health costs by eliminating ineffective therapies, trial-and-error prescribing, and adverse drug effects. Such initiatives require quantitative and functional proteome profiling and dynamic disease biomarkers in addition to current pharmacogenomics approaches. With proteases at the pathogenic center of many diseases, high-throughput protein termini identification techniques such as TAILS (Terminal Amine Isotopic Labeling of Substrates) and COFRADIC (COmbined FRActional DIagonal Chromatography) will be fundamental for individual and comprehensive assessment of health and disease.

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1. The human proteome project on the verge of precision medicine

After the publication of the first draft of the human genome in 2001 by the Human Genome Organization (HUGO) and before its official completion in 2003 [1,2], the Human Proteome Organization (HUPO; www.hupo.org) was founded to promote the field of proteomics and to gain a comprehensive understanding of human biology through mapping of the human proteome. In 2010, the Human Proteome Project (HPP) was announced at the 9th World HUPO Conference in Sydney, Australia, and launched the year after at the World Congress in Geneva, Switzerland. The main goal of the HPP is to identify and characterize at least one proteoform of each of the ~20,000 human protein-coding genes, as well as their co- and post-translational modifications (PTMs). The HPP is organized in a chromosome-centric (C-HPP) manner with each of the 23 human chromosomes and the mitochondrial genome managed by individual member countries to further understand the functions and localization of individual proteins in the human body [3,4]. The biology/disease-centric pillar of the HPP (B/D-HPP) aims to analyze the proteomes of each major human disease to identify and characterize protein perturbations on a systems level [5]. All HPP efforts (Fig. 1A) are assisted by the targeted proteomics resource SRMAtlas [6] and the publicly accessible compendium of identified peptides PeptideAtlas [7], both hosted by the Institute for Systems Biology in Seattle, USA.

Concurrently, the Human Protein Atlas (HPA) program, funded by the non-profit Knut and Alice Wallenberg Foundation, was started in 2003 at the Royal Institute of Technology in Stockholm, Sweden [8]. To date, antibody-based profiling of 44 different tissues, 46 cell lines, and 20 cancer types is completed (Protein Atlas version 13) and complemented by RNA-seq data from 32 different tissues (www.proteinatlas.org). In total, over 13 million manually annotated immunofluorescence-based confocal microscopy images are available, bolstering our knowledge of individual protein expression in human cell lines (The Cell Line Atlas), tissues (The Tissue Atlas), and cancers (The Cancer Atlas), with partial subcellular localization data (The Subcellular Atlas) [9]. Furthermore, in 2014 two mass spectrometry-based drafts of the human proteome were published [10,11], both covering more than 80% of the predicted human proteome, tremendously expanding our current catalogue of expressed proteins in human specimens. But despite squillions of high quality mass spectrometry datasets and a myriad of confocal immunofluorescence microscopy images, more than 2700 protein-coding genes still lack solid evidence at the protein level (neXtProt release 2015-04-28) [12]. Memorably in his 2015 State of the Union Address, US President Barack Obama announced an ambitious plan to invest hundreds of millions of dollars in cutting-edge biomedical research, to launch a new era of medicine, “one that delivers the right treatment at the right time” (Fig. 1B). However, precision medicine, often referred to as personalized or molecular medicine, will be only possible if we know which proteins or more importantly which proteoforms are present and where in the human body during distinct biological events. Thus, further collaborative efforts including “outside of the box” approaches such as positional proteomics will be necessary to set the stage for successful precision medicine initiatives.

2. Positional proteomics to fill the missing proteins gap

Even though modern state of the art mass spectrometers such as the Bruker UHR-QqTOF Impact II [13] and the Thermo Orbitrap Fusion Lumos Tribrid [14] have detection and quantitation limits in the low attomole range, protein concentrations in biological samples span more than 10 orders of magnitude in dynamic range, which hampers the identification of low abundance proteins by conventional shotgun proteomics [15]. In such bottom-up approaches, proteome samples are digested by a highly specific endopeptidase such as trypsin or GluC, and the resulting peptide mixture is analyzed by liquid-chromatography tandem mass spectrometry (LC-MS/MS). Thereby the numerous peptides from abundant proteins such as albumin in plasma, collagen in connective tissue, keratin in skin, or hemoglobin from red blood cells frequently overshadow low abundance peptides typically originating from low abundance proteins, such as cytokines and other cellular mediators. This caveat can be partly overcome by preclearing samples (*e.g.* using immunodepletion kits [16]), protein enrichment (*e.g.* BIO-RAD ProteoMiner [17]), offline sample fractionation [18], or combinations thereof, but these strategies run the risk of losing the low abundance proteins.

Another hurdle is that peptides from some proteins are just not amenable to conventional shot-gun proteomics—peptides are either too short (<7 aa), too long (>30 aa), ionize poorly, or are too hydrophilic or too hydrophobic impeding their handling and online reversed phase (C18) chromatographic separation. Thus shotgun proteomics using a single endopeptidase for proteome digestion is frequently insufficient to comprehensively and unambiguously characterize the human proteome [19]. Positional proteomics techniques such as TAILS (Terminal Amine Isotopic Labeling of Substrates; Fig. 2A) and COFRADIC (COmbined FRActional DIagonal Chromatography; Fig. 2B) not only deplete internal endopeptidase-generated peptides and thus simplify the peptide-mixture, but also focus on protein N- or C-terminal peptides and protease-cleaved neo-termini with altered physicochemical, *m/z*, ionization, and fragmentation properties compared to their shot-gun counterparts since they are semi-specific, *i.e.* unlike shot-gun peptides, they only have one terminus corresponding to the chosen endopeptidase cleavage site (*e.g.* trypsin KR) [20,21]. Thus terminomics is the perfect complement to conventional shot-gun proteomics to fill the missing proteins gap, especially when specialized cells (*e.g.* platelets, erythrocytes) or rare human specimens (*e.g.* dental pulp, heart) are targeted [20,21]. More than 12% of canonical human proteins have yet to be detected by mass spectrometry (neXtProt release 2015-04-28), an astonishing and somewhat surprisingly high number considering the tremendous efforts of the proteomics community in the last 10 years. Hence new approaches are needed to take on the challenge, *e.g.* by using TAILS N-terminomics on human dental pulp, we identified >4000 different proteins, including 174 proteins that had not been identified by any other proteomics approach before [22].

3. High-throughput terminomics techniques

COFRADIC was the first positional proteomics technique to use negative selection and a series of orthogonal liquid chromatographic separations to enrich for protein N-termini prior to MS/MS analysis [23]. After reduction and alkylation of cysteine side chains and acetylation of all primary amines (N-terminal α-amines and lysine ε-amines) at the intact protein level, proteome samples are digested with trypsin or any other highly specific endopeptidase to generate (i) N-terminally acetylated original protein N-termini, and (ii) internal peptides exhibiting free α-amines. Peptides representing protein N- and C-termini are enriched by a strong cation exchange chromatography (SCX) step and recovered in the flow-through due to their experimentally introduced or inherently lower basicity, respectively, whereas internal peptides interact with the resin and elute later corresponding to their positive charge and/or higher basicity [24]. Next, two orthogonal reversed-phase liquid chromatography (RP-HPLC) runs are performed with 2,4,6-trinitrobenzenesulfonic acid (TNBS) treatment of the fractions

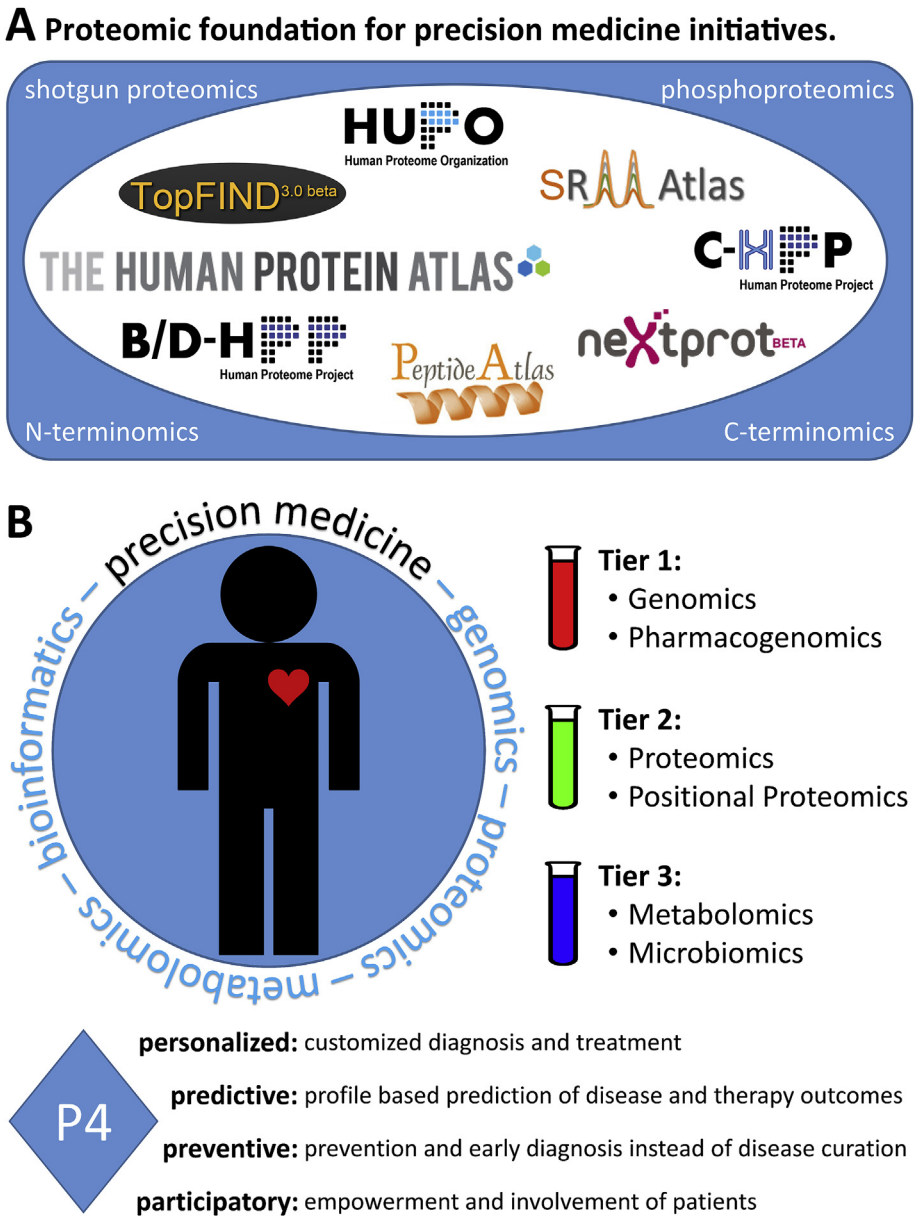

Fig. 1. "Precision Medicine" rests upon genomics, proteomics, metabolomics, and bioinformatics. (**A**) Collaborative efforts providing the foundation for proteomics-based precision medicine initiatives are highlighted. Data repositories such as Peptide Atlas and knowledgebases like neXtProt and TopFIND are key to disseminate data and knowledge to the broader scientific community, providing the foundation for the development of diagnostic, prognostic, therapeutic, and preventive medical applications. **HUPO**: the Human Proteome Organization represents and promotes proteomics to gain a deeper understanding of human disease and all other aspects of human well-being. **C-HPP**: the aim of the chromosome-based Human Proteome Project is to characterize all ~20,000 protein-coding genes to generate a human proteome map as a resource to elucidate biological and molecular functions and to advance disease diagnosis and treatment. **B/D-HPP**: the Biology/Disease Human Proteome Project aims to expand our current understanding of the human proteome and to provide a spectrum of research tools for studying the proteins of relevance in specific areas of biology or disease. **The Human Protein Atlas** explores the human proteome using a combined antibody-transcriptomics approach. In the current version (v13), transcriptomes of 213 tissues and cell lines and proteome analyses based on >24,000 antibodies are publically available. **PeptideAtlas** is a publically available, multi-organism compendium of peptides identified in a large set of proteomics experiments, all analyzed in a uniform manner. **SRMAtlas** focuses on targeted proteomic assays to detect and quantify proteins in complex biological samples by selected/multiple reaction monitoring (SRM/MRM). **neXtProt** serves as a comprehensive human-centric discovery platform, expanding SwissProt protein information by adding high quality data from a variety of high-throughput approaches such as microarrays, antibody screens, and proteomics. **TopFIND$^{3.0}$** is the go-to knowledgebase and analysis resource for protein termini and proteolytic processing, covering more than 290,000 protein N- and C-termini, with more than 33,000 annotated protease cleavage sites. (**B**) Future medicine is envisioned as **p**ersonalized, **p**redictive, **p**reventive, and **p**articipatory (P4 medicine), and thus relies on individual molecular profiles assembled from genomics, proteomics, and other omics-based approaches. Current P4 initiatives build mostly on pharmacogenomics (Tier 1), but due to the inherent static nature of genomic information, dynamic processes simply cannot be monitored. Thus proteomics and terminomics-oriented approaches (Tier 2) gain importance and are facilitated by large-scale collaborative efforts such as the Human Proteome Project (HPP) and The Human Protein Atlas. Other omics-techniques such as metabolomics and microbiomics (Tier 3) complement the molecular profiles.

between runs to increase the hydrophobicity of co-enriched C-terminal and internal peptides, thus dramatically changing their retention time and allowing their depletion. Only fractions containing peptides representing original protein N-termini are then analyzed by LC-MS/MS (Fig. 2B). A recent variation of this technique (ChaFRADIC) has improved efficiency of peptide identification and requires less protein for analysis [25].

TAILS N-terminomics was initially designed for protease substrate discovery [26,27] and subsequently adapted to monitor *in vivo* proteolytic processing and perturbations of the protease

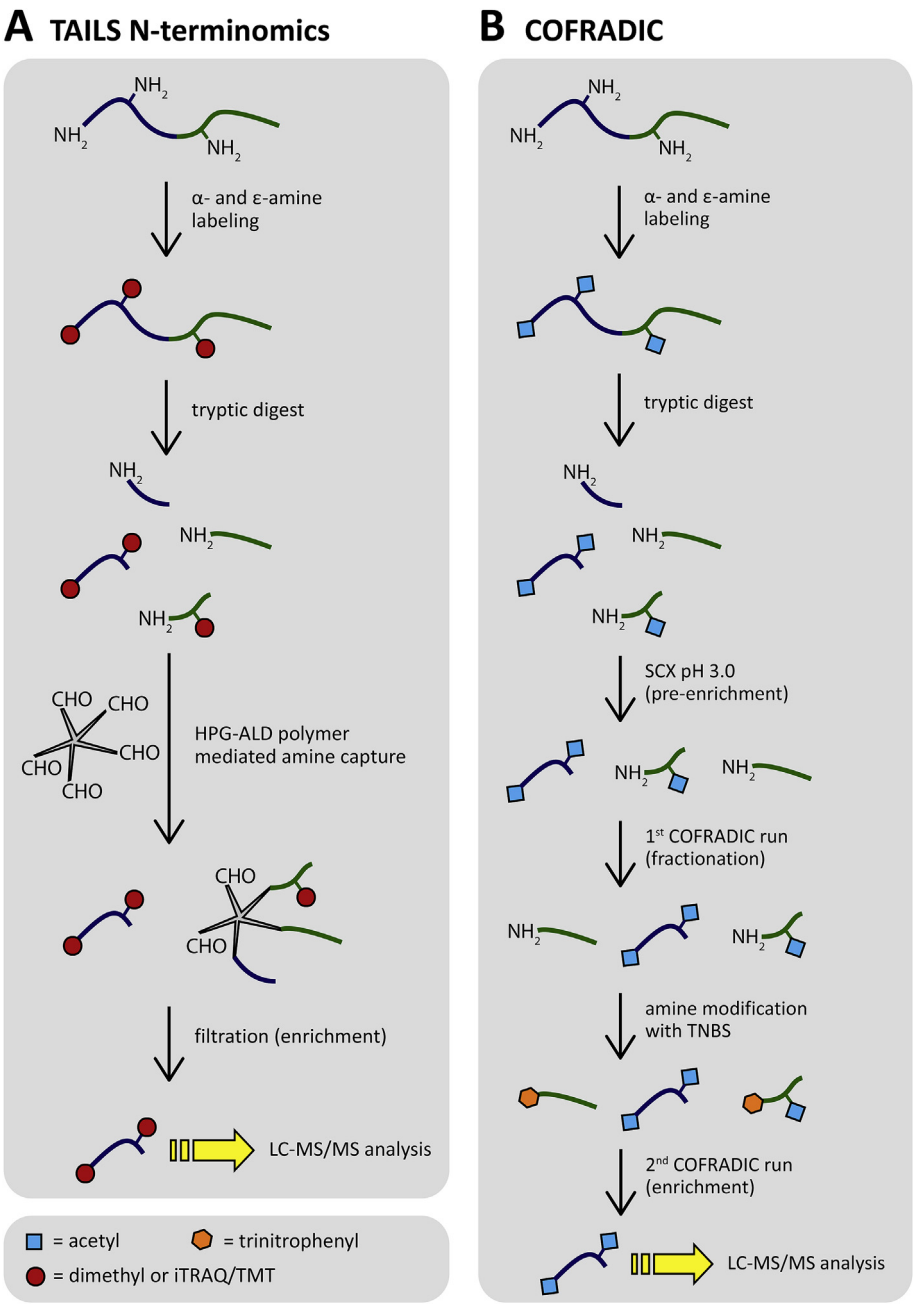

Fig. 2. Schematic representation of the two most prominent N-terminal negative enrichment strategies in positional proteomics: **(A)** TAILS and **(B)** COFRADIC. N-terminal peptides are enriched by selective removal of internal and C-terminal peptides following primary amine protection at the protein level and sample digestion *e.g.* by trypsin. Importantly, negative enrichment strategies also enrich for naturally blocked α-amines, and thus allow the study of *in vivo* protein acetylation profiles. Please refer to main text or more methods oriented reviews [36,38] for details.

web in health and disease [28–30]. Cysteine side chains and primary amines are blocked at the protein level before proteome digestion, typically by trypsin or GluC, allowing the analysis of both natural N-termini and *in vivo* generated neo-N-termini. As formaldehyde or isobaric tags are used for amine blocking, naturally blocked N-termini can easily be differentiated from internal, typically trypsin-generated peptides, and acetylation profiles of protein N-termini can be studied on a systems level [20,21]. Following protein digestion with trypsin or any other highly specific endopeptidase, the peptide mixture is incubated with a commercially available, water-soluble, aldehyde-derivatized (HPG-ALD) 100-kDa polymer (http://flintbox.com/public/project/1948/). HPG-ALD polymer covalently binds trypsin (or GluC) generated internal peptides that have reactive free α-amines. As naturally and experimentally blocked N-termini are unreactive, they are easily separated from the polymer-bound internal peptides by ultrafiltration prior to LC-MS/MS analysis [26,31]. For academic users, the HPG-ALD polymer is currently available for USD350 per 20 mg (retrieved 2015-10-23), which allows TAILS analysis of a total of 4 mg proteome (4–8 TAILS experiments) (Fig. 2A).

Recently two similarly elegant N-terminomics approaches were published based on charge reduction [32] or charge reversal [33] of internal peptides in combination with SCX, and additionally, there are positive enrichment methods such as N-CLAP (N-terminomics

by Chemical Labeling of the α-Amine of Proteins) [34] or subtiligase-based approaches [35]. Although the latter approaches cannot assess natural N-terminal modifications as a free α-amine is needed for tagging and pullout, both are suited to analyze protease-generated neo-N-termini [36–39].

C-terminomics is inherently complicated due to the low chemical reactivity of carboxyl groups. Nevertheless, modified TAILS [40] and COFRADIC [41] protocols allow for high-content analysis of protein C-termini [37,42]. Due to more complex and labor-intensive workflows and lower success rates, C-terminomic approaches are less frequently used than their N-terminomic counterparts. However, improved protocols are in development with simplified chemistry and improved depletion polymers that will allow the scientific community to adopt C-terminomics into their day-to-day proteomics repertoire [37]. In addition to the lower chemical reactivity, both natural and neo-C-termini lack a basic residue after trypsin digestion and hence are often missed by LC-MS/MS analysis. To counter this, we added the trypsin-mirroring metalloprotease LysargiNase from the thermophilic archaea *Methanosarcina acetivorans* to the proteomics tool box [43]: C-termini generated by LysargiNase carry a N-terminal lysine or arginine, resulting in high quality, b-ion dominated MS/MS spectra. In addition, LysargiNase cleaves before mono-, di- and trimethylated lysine and arginine residues facilitating detection of epigenetic histone modifications, and increases proteome coverage especially when used in combination with trypsin. Crystallization-grade LysargiNase is available for less than USD400 per mg of protease (http://www.ibmb.csic.es/LysargiNase/; retrieved 2015-10-23), bringing the costs of a typical 100 μg proteome digest to less than a dollar.

4. Proteolytic signatures as candidate biomarkers and positional proteomics for precision medicine

4.1. Proteolysis—a many-faced phenomenon

With over 560 human proteases, it is not surprising that proteolysis plays a pivotal role in most physiological and pathological processes. Unlike other PTMs such as protein glycosylation or phosphorylation, proteolysis is an irreversible mechanism for achieving precise cellular control of biological processes [44]. But reversible exceptions exist: A few proteases, such as the asparaginyl endopeptidase legumain, have tightly controlled dual protease–ligase activities, complicating the picture by reversing their initial cleavages, or by engaging in *in cis* or *in trans* protein splicing and thus generating even more proteoforms [45]. Most secreted proteins undergo proteolytic signal peptide removal in the endoplasmic reticulum (ER) before they are released to the extracellular space. Many proteases are synthesized as inactive zymogens that require proteolytic activation to prevent inadvertent activity as well as having activity blocked by endogenous inhibitor binding. For example, matrix metalloproteinases (MMPs) are central players in a myriad of physiological processes and signaling events especially in the extracellular matrix of connective tissues, and thus require regulation at all levels during their lifetime. Thus, besides their tight control at the transcriptional level and their expression as proenzymes, MMPs are also actively controlled by the endogenous tissue inhibitors of metalloproteinases (TIMPs) 1 to 4 [46,47].

Bioinformatic protease web analysis recently verified that regulatory crosstalk occurs between proteases—either by direct cleavage or by targeting the corresponding inhibitors, independent of protease class or classical biochemical cascades [28]. Consequently, proteases cannot be viewed in isolation and must be examined together with their modulators and substrates (Fig. 3): For example, MMP8 cleaves the elastase inhibitor α1-antitrypsin, releasing the brake from neutrophil elastase and thus triggering the activating cleavage of CXC chemokine 5 (CXCL5, LIX) by elastase *in vivo*. N-terminally processed CXCL5 binds to its cognate receptor CXCR2, enhancing intracellular calcium mobilization and neutrophil chemotaxis. On the contrary, MMP12 cleavage inactivates CXCL5 and shuts down the neutrophil response [28,48].

Another example of the interplay between endogenous inhibitors and MMPs in the protease web is described for the complex activation of MMP2 by membrane-type 1 MMP (MT1-MMP, MMP14) at the cell surface, which requires appropriate stoichiometric concentrations of furin-activated MT1-MMP and TIMP2 to form a 1:1 complex [49]. This inactive complex then recruits proMMP2 from the extracellular space to the cell surface to form a ternary complex, in which MMP2 interacts via its C-terminal hemopexin domain with the C-terminal domain of TIMP2. An adjacent uninhibited MT1-MMP molecule then partially activates the complexed proMMP2 by cleaving between Asn66 and Leu67. Further processing *in trans* by an active MMP2 molecule between Asn109 and Tyr110 produces fully activated MMP2 that is released from the complex to the extracellular space or sequestered at the cell surface by binding to a membrane docking protein such as the vitronectin receptor αVβ3 or collagen [50]. However, if the concentration of TIMP2 is high, both MMP2 and MT1-MMP are inhibited and no MMP2 activation occurs [49]. Due to its pleiotropic roles, MMP2 has been described as both a drug target and anti-target, depending on the cellular context, tissue, disease type and duration, and the stage of immune cell infiltration [51]. Thus, in most cases, neither an individual protease activity nor a single substrate cleavage will delineate the health state of a tissue or patient - instead, an ensemble of protein cleavages and associated protein neo-N-termini that forms a “proteolytic signature”, will likely provide the required complexity, sensitivity, and robustness to comprehensively define a patient's condition.

4.2. Biomarker discovery

We recently proposed protein termini as new prospects for biomarker discovery [52], as even though thousands of shot-gun proteomics-derived biomarkers have been suggested for cancer and other diseases, their translation into the clinic has been unexpectedly disappointing [52,53]. Disease-specific proteolytic processing occurs in most pathologies including inflammation [29] and cancer [54], and importantly, perturbations of the protease web are often associated with disease initiation and change upon disease progression and manifestation [55,56]. Ensembles of characteristic protein termini with qualitative differences in both intact and neo-termini form a highly informative proteolytic signature—they reflect the mechanistic state of bioactive proteins and in many cases their cellular localization, potentially allowing the discrimination between health and disease and even disease stages [52]. A prominent example of the importance of protein N-termini are chemokines: After proteolytic signal peptide removal in the ER and secretion into the extracellular space, chemokines create a haptotactic gradient and induce directed chemotaxis in nearby responsive cells. However, precise proteolytic processing by MMPs modulates chemokine receptor specificities and/or turns receptor agonists into antagonists and *vice versa*, manipulating the cellular response [57,58]. Similarly, chemokine regulation has been recently observed by cysteine cathepsins, which activate ELR-positive CXC chemokines (*e.g.* interleukin-8), but inactivate non-ELR chemokines such as SDF-1 (stromal cell-derived factor 1, CXCL12) [59]. Thus, it is of tremendous importance to know a chemokine's N-terminus before assessing the health state of a patient. Similarly,

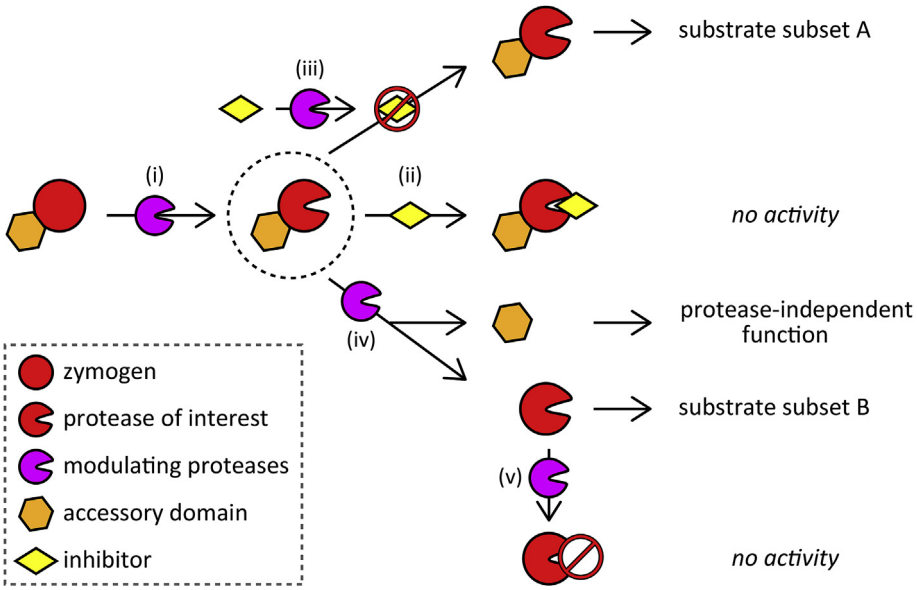

Fig. 3. Proteolytic networks determine the fate of bioactive molecules. Proteolytic enzymes are regulated on several levels: (i) Typically synthesized as inactive zymogens, proteolytic cleavage is needed for activation; (ii) after activation endogenous inhibitors control their activity; (iii) inhibitor cleavage by another protease relieves the inhibition and initiates *downstream proteolysis*; (iv) removal of protein interaction or localization-mediating accessory domains from a protease can alter the substrate repertoire and thus the biological effects of that protease, explaining why many proteases are found as both drug targets and anti-targets depending on the disease and tissue context; (v) inactivating cleavages accelerate the degradation of proteases to remove these from the proteolytic network when their task is over. For simplicity reasons, the figure only shows the regulatory levels for one protease (protease of interest; shown in red), but similar checkpoints are in place for all other indicated proteases.

proteolytic processing removes the first two alpha-helices of 14-3-3 proteins that are indispensable for dimerization, rendering them monomeric and altering their function [20,60]; or in case of the endogenous serine and cysteine protease inhibitors, serpins and cystatins, only the identification of either natural or neo-N-termini discloses if the inhibitor is active or proteolytically inactivated respectively [29,61].

N-terminal modifications [37] are as important as neo-N-termini in assessing the bioactive state of molecules, and thus will be valuable contributors to proteolytic signatures. For example, pyroglutamate formation and acetylation can be crucial for protein function: N-terminal pyroglutamate formation in beta-amyloid increases its hydrophobicity and resistance to aminopeptidases, resulting in more aggregation and plaque formation in Alzheimer's disease; N-terminal acetyltransferases have been implicated as both oncogenes and tumor suppressors in human cancer survival and proliferation since N-terminal acetylation modulates pathways in apoptosis, cell-cycle arrest, and autophagy in a cancer-dependent manner [62–64].

In 2014, a circulating proteolytic signature of cell death was discovered in plasma samples from cancer patients by N-terminomics [56], allowing the monitoring of chemotherapy treatment and its efficacy. Despite its complex nature and the high abundance of albumin, plasma is still the biological fluid of choice in clinical applications as it is easily obtained, shows lower variability than serum due to minimal sample handling, and provides a reflection of a patient's health [15,53,65]. As many proteolytic processes occur in the extracellular environment, both neo-N and neo-C-termini are traceable in plasma. However, other clinically accessible fluids, such as urine or saliva, may be of advantage in certain cases and should be tested in parallel during biomarker development [52,66]. Even though clinical evaluation of proteolytic signature biomarkers will take place in readily available biofluids, their initial characterization will most likely originate from in depth comparisons of pathologically affected versus healthy tissues, which are then translated in Multiple Reaction Monitoring (MRM) type assays into the clinic [52]. Along these lines, we recently published a targeted MS/MS method based on Proteolytic Signature Peptides (PSPs), which include the tryptic peptide spanning the cleavage site in the intact protein, and the semi-tryptic peptides representing the respective neo-N- and neo-C-terminus after *in vivo* cleavage [67]. Thus, generation of cleaved neo-termini is mirrored by a reduction in the amount of the spanning tryptic peptide in the uncut protein. Once these proteolytic signature peptides are established, the amount of proteolytic processing can even be quantified using chemically synthesized and isotopically-labeled counterparts as internal standards (Fig. 4). Such peptides can also be translated as SWATH-detected peptides [68–70] in contemporary high throughput, high content approaches.

4.3. Precision medicine: present and future

Genomics is without a doubt the current key technology in personalized medicine (Fig. 1B). The newest generation Illumina sequencer [71] allows for complete genome sequencing in less than a day, and individual genome sequencing for less than 1000 USD, an incredible cost reduction considering that the first sequenced human genome in 2003 came with a price tag of around 3 billion USD. But in order to obtain a thorough understanding of how certain genes or gene sets really correlate with health and disease, thousands and thousands of 1000 USD genomes will have to be sequenced with follow-up data analysis at a combined cost of >10 billion dollars [71,72]. Despite these enormous efforts, there is some doubt whether classical genomics alone will ever be able to explain non-monogenic and thus more complex diseases such as type 2 diabetes, cancer, or allergic and autoimmune disorders, as the genetic sequence *per se* does not change over time. However, proteomes constantly respond to all kind of changes, whether they are small (*e.g.* dietary) or large (*e.g.* viral infection) [73,74]. In fact many of today's top selling drugs

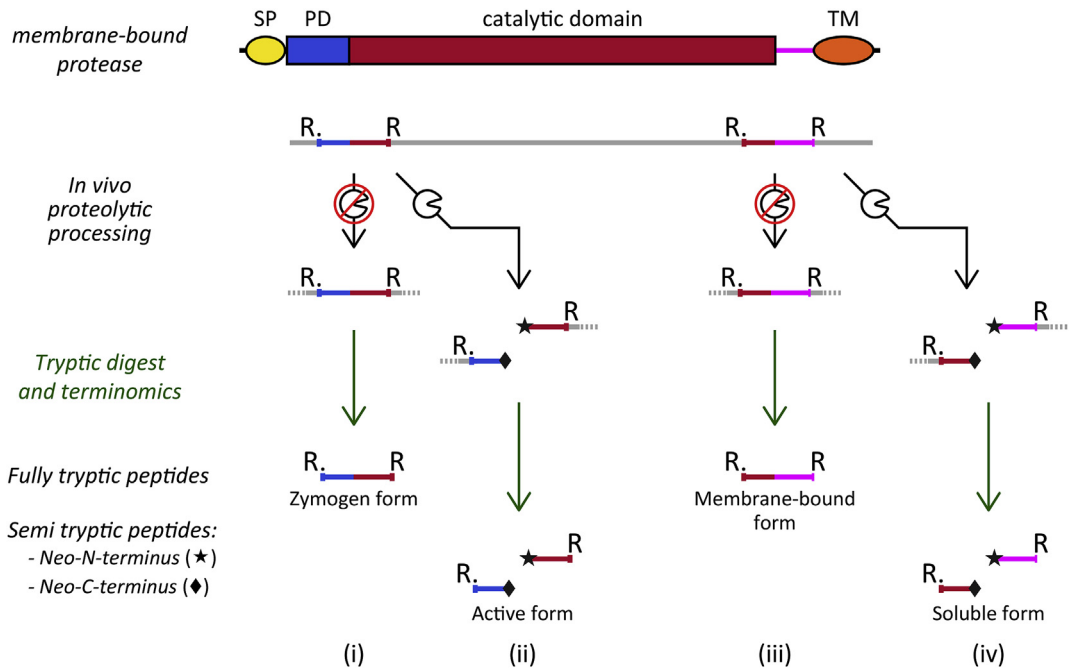

Fig. 4. Proteolytic Signature Peptides (PSPs) characterize bioactive molecules. Proteomics, but especially N-terminomics, allows for the identification and quantification of proteolytic processing by targeting: (i) and (iii) a tryptic peptide spanning a potential protease cleavage site that has not been cut - designated a "spanning peptide"; (ii) and (iv) the corresponding N- and C-terminal semi-tryptic peptides which have been cut—thus differentiating between the intact protein and the proteolytically processed variant. Thus, PSPs can delineate inactive *vs.* active or membrane-bound *vs.* soluble proteoforms of membrane-bound proteins such as matrix metalloproteinases *e.g.* Mt1-MMP (MMP14), or can distinguish CXC chemokine receptor agonists from antagonists. By implementing isotopically-labeled counterparts as internal standards, absolute quantitation of *in vivo* proteolytic processing is possible. SP, signal peptide; PD, prodomain; TM, transmembrane domain.

help less than 25% of patients and in case of the cholesterol-lowering HMG-CoA reductase inhibitors, as few as 2% of patients actually benefit from the drug treatment—highlighting the pressing need for personalized approaches [75]. Without a doubt, genetic analysis and pharmacogenomics [76–80] are an important step in the right direction, but we are convinced that proteomic insights and quantitative protein biomarkers will be needed to reliably characterize and longitudinally monitor individuals, their disease courses and therapeutic responses. Collaborative proteomic efforts are already creating a flux of "big data" and knowledge on life-threatening diseases (Fig. 1A). But most likely, only a combination of cumulative single person studies following the same study design and well-designed mobile applications enabling people to monitor their personal health in between doctor's visits will allow true precision medicine with proteomics as the central diagnostic tool [75,81]. Positional proteomics will add the extra layer of information needed to understand and interpret the dynamic proteolytic networks underlying health and disease, eventually permitting the prescription of the right drug at the right time and dose to the individual patient.

4. Conclusion

Since the launch of the Human Proteome Project in 2011, proteomics has been established together with pharmacogenomics as the future direction for precision medicine, and positional proteomics is now poised to make a unique contribution for earlier and improved diagnoses, personalized drug prescriptions, reduced inappropriate therapeutic interventions, and a better health care system. The importance of properly adjusted proteolytic networks cannot be overestimated in health. Therefore, attention should be paid to perturbations and the resolution of proteolytic *status quo* when monitoring disease initiation, progression and therapeutic responses.

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