

REVIEW

Understanding fibroblast activation protein (FAP): Substrates, activities, expression and targeting for cancer therapy

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Fibroblast activation protein (FAP) is best known for its heightened expression in tumour stroma. This atypical serine protease has both dipeptidyl peptidase and endopeptidase activities, cleaving substrates at a post-proline bond. FAP expression is difficult to detect in non-diseased adult organs, but is greatly upregulated in sites of tissue remodelling, which include liver fibrosis, lung fibrosis, atherosclerosis, arthritis, tumours and embryonic tissues. Due to its restricted expression pattern and dual enzymatic activities, FAP is emerging as a unique therapeutic target. However, methods to exploit and target this protease are advancing more rapidly than knowledge of the fundamental biology of FAP. This review highlights this imbalance, emphasising the need to better define the substrate repertoire and expression patterns of FAP to elucidate its role in biological and pathological processes.

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1 Introduction

Proteolysis is an irreversible and essential post-translational modification that controls the composition and activity of proteins in a biological system. Bioactive peptides influence a range of physiological processes, including glucose homeostasis, the immune response and cellular signalling, and modification of these peptides can have significant impacts on such processes. Identifying proteases and how they modify

their substrates is imperative in understanding cell biology, physiology and pathogenesis and in determining the potential of these proteases as drug targets.

The dipeptidyl peptidase (DPP) 4 protein family has four enzymatic members; DPP4, fibroblast activation protein (FAP), DPP8 and DPP9 (Fig. 1), which are able to hydrolyse a prolyl bond that is two amino acids from the N-terminus of a protein. Due to the cyclic nature of the proline (Pro) residue, this is a rare catalytic ability that makes this group of proteases interesting in many aspects of biology and as therapeutic targets [1, 2].

DPP4 is the most profoundly characterised member and prototype of this family; it is expressed ubiquitously and has a role in a wide range of physiological and pathological processes via its cleavage of bioactive peptides [1]. DPP4 was identified as a drug target for type 2 diabetes after it was observed to cleave and inactivate glucagon-like peptide-1 *in vivo*, thereby muting insulin secretion by the pancreas. This is just one example of how an understanding of the substrates of a protease can provide crucial information on its physiological role and thus lead to a new therapy.

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Abbreviations: α_2 -AP, α_2 -antiplasmin; APP, amyloid precursor protein; CAR, chimeric antigen receptor; CN, collagen; DPP, dipeptidyl peptidase; ECM, extracellular matrix; FAP, fibroblast activation protein; NPY, neuropeptide Y; PREP, prolyl endopeptidase; SRM, selected reaction monitoring; TAILS, terminal amine isotopic labelling of substrates; TG, thapsigargin

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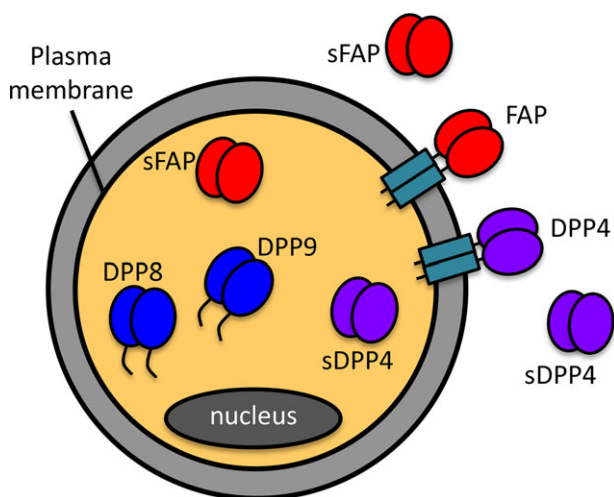


Figure 1. Enzymatic members of the DPP4 family. FAP and DPP4 are type II integral membrane proteins that have intracellular and extracellular soluble, truncated forms. DPP8 and DPP9 are intracellular proteins.

FAP is closely related to DPP4, sharing 52% amino acid sequence identity [3]. However, unlike DPP4, the substrate repertoire and the patterns of *in vivo* FAP expression are poorly defined. Despite this, FAP is attracting attention in cancer, cardiology and fibrosis research because its expression is greatly upregulated in disease. A number of tools have thus been developed to target and exploit this protease for therapeutic intervention. The aim of this review is to emphasise the need to elucidate the roles and functions of this protease through substrate discovery in order to fundamentally progress the field.

2 Fibroblast activation protein

2.1 Enzyme activity and substrate specificity

Unique from other members of the DPP4 family, FAP has both DPP and endopeptidase activities (Fig. 2). However, despite exhibiting both activities, FAP has been shown to be a more efficient endopeptidase than DPP [4, 5]. The activities and specificities of FAP have been investigated using artificial substrates and synthetic peptide libraries [4, 6–9], which revealed a strong preference for FAP cleavage of endopeptidase substrates after glycine-proline (Gly-Pro) motifs [4, 8, 9]. This restricted cleavage site specificity was supported by results obtained using a fluorescence resonance energy transfer substrate library, which also identified endopeptidase cleavage events only after Gly-Pro motifs [8]. However, a MS-based analysis of FAP endopeptidase specificity using gelatin as a substrate proposed that cleavage can also occur C-terminal to Ala-Pro, Arg-Pro, Lys-Pro and Ser-Pro motifs as well as the reported Gly-Pro motif [10]. The disparate results from these cleavage site specificity profiling studies highlight the

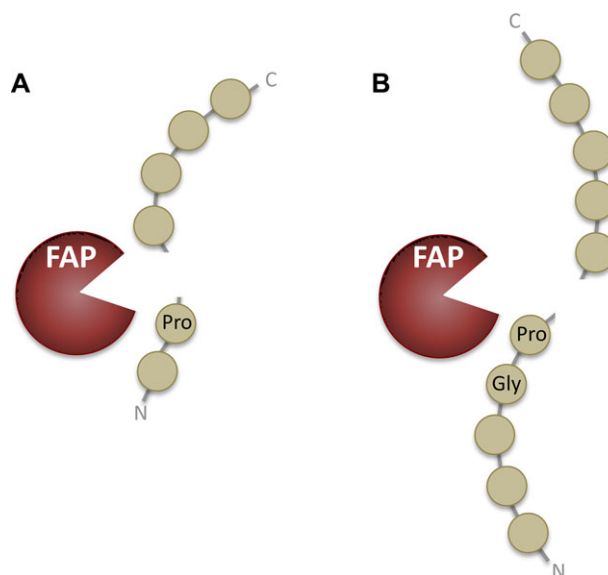


Figure 2. Dual-enzyme activity of FAP. (A) Dipeptidyl peptidase activity of FAP allows it to cleave two amino acids off the N-terminus of a protein. This cleavage occurs after a proline (Pro) residue. (B) Endopeptidase activity of FAP enables cleavage that is more than two amino acids from the N-terminus of a protein. Cleavage is restricted to the post-Pro bond after glycine-proline (Gly-Pro) (adapted from [68] with permission).

limitations of such biochemical approaches. Synthetic peptide libraries do not take into account native protein or peptide structure or the contribution of exosites in protease–substrate interactions. Thus, identification of novel physiological FAP substrates will assist in defining the cleavage site specificity of FAP and determining the potential contribution of exosite interactions of this protease during cleavage events.

2.2 Substrates *in vitro* and *in vivo*

Although FAP is involved in numerous (patho-) physiological processes, its substrate repertoire remains mostly unknown. However, screening known DPP4 substrates against a recombinant human soluble form of FAP has identified a number of natural FAP substrates [11]. In that study, full-length neurological peptides were cleaved by FAP and analysed by MALDI-TOF-MS. Four neuropeptides were efficiently cleaved by FAP, namely neuropeptide Y (NPY), peptide YY, B-type natriuretic peptide and substance P, with *in vitro* half-lives comparable to incubation with DPP4 [11]. Those results clearly show that the DPP specificity of FAP is much less restricted than previously thought, with cleavage occurring after Lys-Pro, Tyr-Pro, Arg-Pro and Ser-Pro dipeptides [11]. Interestingly, DPP cleavage was not observed for some other potential peptide substrates that were also screened and had the same N-terminal dipeptide sequence as the four cleaved neuropeptide substrates. Furthermore, in a previous study using a synthetic dipeptide fluorescent substrate library, limited levels of cleavage by FAP

were reported for these particular dipeptide sequences [6]. Together, these data strengthen the notion that exosite binding has a role in FAP cleavage events.

As the endopeptidase activity of FAP distinguishes this protease from other members of the DPP4 family, it could be speculated that this may be its predominant enzymatic role as well as its unique role. Towards this, two physiological endopeptidase substrates have so far been discovered for FAP; denatured type I collagen (CN-I) [12, 13] and α_2 -antiplasmin (α_2 -AP) [14, 15]. In both cases, cleavage occurs after a Gly-Pro motif [10, 14].

CN fibres are major components of the extracellular matrix (ECM), providing structural support for cells and tissues. ECM proteins also bind and sequester growth factors and bioactive peptides, thus regulating and influencing important biochemical and biomechanical processes [16]. As CN is rich in Gly-Pro residues, it is unsurprising that FAP is able to digest this ECM protein. While FAP is unable to cleave CN-I in its native form, partial digestion, for example, by matrix metalloproteinase 1, results in unwinding of the CN fibre, and this facilitates FAP cleavage [12, 17]. FAP is also able to cleave denatured CN-III in vitro [17]. CN deposition and degradation in ECM remodelling are common processes in tumourigenesis and fibrosis. The ability to recognise and digest denatured CN-I implicate FAP in ECM remodelling and the pathological processes where this is abnormal.

As well as being a cell surface protease, FAP also exists in a truncated, soluble form in human plasma, lacking the transmembrane domain [15, 18]. Soluble FAP cleaves α_2 -AP at prolyl bonds Pro3-leucine4 and Pro12-Asn13 [18]. During tissue repair, fibrin is deposited to form a fibrin clot. Fibrinolysis is the natural process in which a fibrin clot is dissolved by plasmin leading to scar resolution. α_2 -AP is an inhibitor of plasmin and therefore reduces the rate of lysis of the fibrin clot (Fig. 3). However, cleavage of α_2 -AP by FAP converts α_2 -AP into a more potent inhibitor of plasmin [18]. We have therefore hypothesised that this activity of FAP leads to a reduction in fibrinolysis and promotion of scar formation in tissues [1].

2.3 Expression pattern

FAP is a difficult protein to study due to the limited availability of specific or selective tools to detect and target it [19]. However, recent developments have enabled more accurate and thorough characterisation of the expression pattern of this protease.

Initially it was widely accepted that FAP was either not expressed or present at insignificant levels in normal adult tissue. However, under certain biological circumstances, FAP expression has been observed, such as during mouse embryogenesis [20] and in the resorbing tadpole tail [21]. These observations suggest a role for FAP in normal developmental processes and tissue remodelling. Despite the observed expression of FAP in healthy tissue and its presence

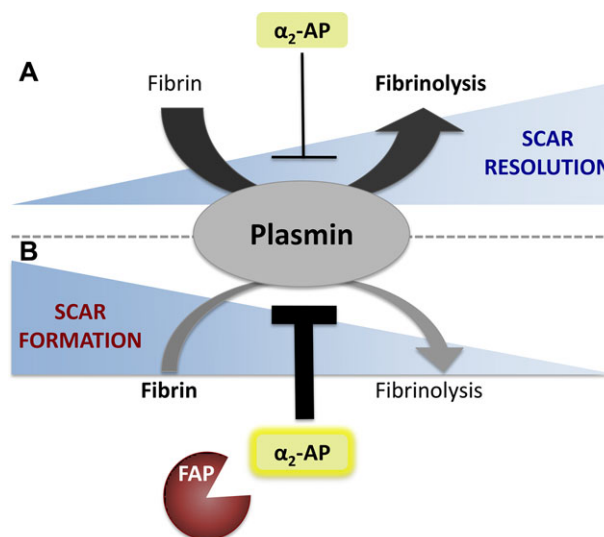


Figure 3. FAP modulates fibrinolysis. α_2 -Antiplasmin (α_2 -AP) becomes a more potent inhibitor of plasmin following FAP-mediated cleavage between Pro12 and Asn13. This results in a shift from increased fibrinolysis and scar resolution in the absence of FAP (A), to decreased fibrinolysis and increased scar formation when FAP is present (B) (adapted from [1] with permission).

in plasma, the FAP knockout mouse has a healthy phenotype [22, 23], suggesting that the role of this protease under normal circumstances is redundant or non-essential.

FAP expression is, however, implicated in a number of pathological processes, resulting in the emergence of this protease as a potential therapeutic target. FAP expression has been observed during wound healing [24], at sites of inflammation including arthritis [25, 26] and in atherosclerotic plaques [27]. Greatly increased intrahepatic FAP expression occurs in cirrhosis and correlates with liver fibrosis severity, where it is expressed by activated hepatic stellate cells [13, 28–30]. FAP is also strongly expressed by stromal fibroblasts in over 90% of epithelial carcinomas [31]. However, despite efforts to study this protease in various cancer models [32–35], little is known about its enzymatic role in the tumour microenvironment. It is often speculated that the collagenolytic role of FAP promotes invasion of tumour cells. Supporting this idea, FAP-positive fibroblasts isolated from human breast tumour stroma co-cultured with breast cancer cells lead to increased cancer cell migration and induction of epithelial–mesenchymal transition [36]. FAP-expressing cells have been shown to have an immunosuppressive role in mice in an immunogenic cancer model [37]. In that study, depletion of FAP-expressing cells resulted in rapid hypoxic necrosis of tumour and stromal cells. The enzymatic or extra-enzymatic mechanism by which FAP is involved in these processes requires further investigation.

Several studies have linked FAP to promoting angiogenesis in the tumour microenvironment [34, 38, 39]. One such study demonstrated that inhibition of both DPP4 and FAP led

to decreased tumour vascularisation, but such a decrease was not observed when DPP4 alone was inhibited [34], thus implicating the enzyme activity of FAP in promoting angiogenesis. FAP is able to cleave NPY to generate a truncated form [11] that is proangiogenic [40], which indicates that increased vascularisation may be mediated partly by FAP cleavage of NPY.

The persistent expression of FAP in tumour stroma clearly implicates this protease in tumourigenesis [31]. However, there is controversy over this role for FAP, with some studies correlating elevated FAP expression with tumour suppression, while others show a correlation with tumour progression [33]. An important consideration when interpreting results from such studies is the source of FAP expression. Although it is well established that FAP is expressed by stromal cells [31], particularly at the invasive front [36, 41], many studies that aim to investigate this protease in the tumour microenvironment have focussed on cancer models using tumour cells that artificially express FAP [42], which is not an accurate reflection of the *in vivo* tumour microenvironment and results from such studies should be interpreted cautiously. In spite of this, the role of FAP in tumour stroma is still highly contextual and further investigation is required to elucidate its function in tumourigenesis.

In addition to the strong focus on FAP as a tumour stroma marker, FAP is also considered to be a protease of interest in several other pathological conditions, including atherosclerosis [27, 33], where FAP enzyme activity has been implicated via cleavage of CN-I, promoting plaque instability [27]. In this condition, FAP is expressed in human aortic smooth muscle cells and FAP expression correlates with progression of atherosclerotic plaques. Moreover, in this study, higher FAP levels were detected in thin fibrous caps compared to thick caps and treating human sclerotic plaques *in vitro* with FAP enzyme activity neutralising antibodies decreased FAP-associated CN degradation [27]. Thus, the collagenolytic activity of FAP may be an important driver of fibrous cap rupture. *In vivo* studies are needed to further evaluate FAP as a candidate therapeutic target for the treatment of patients with high-risk atherosclerotic plaques.

2.4 FAP as a biomarker

A major hurdle in the study of FAP as a potential disease biomarker is the inability to specifically measure FAP enzyme activity. However, an FAP-specific substrate has recently become available, enabling the measurement of FAP activity in fluids and organs from diseased and non-diseased samples [43]. There is a clear need to closely examine whether FAP enzyme activity levels associate with disease progression and pathogenesis to better understand roles of FAP, and to determine whether an FAP-specific enzyme assay could be an informative diagnostic tool in the clinical setting. For example, such an assay may be useful for selecting patients who may be more responsive to a FAP-targeted therapy.

However, regardless of whether FAP itself correlates with severity of disease or tumour progression, the ratio of full-length to cleaved FAP substrates may be found to change in relation to pathogenesis. If so, FAP substrates might become disease biomarkers. Consider the case of FAP-mediated cleavage of NPY, where the resulting cleaved form of NPY is proangiogenic [40]. In the normal healthy adult, FAP digestion of NPY may be negligible and potentially redundant as DPP4 is ubiquitous and cleaves NPY efficiently. However, in a patient with an aggressive epithelial tumour, the stromal fibroblasts surrounding the tumour can express high levels of cell surface FAP and thus the contribution by FAP to the N-terminal cleavage of NPY in that microenvironment may supersede the contribution of DPP4. Thus, levels of cleaved versus full-length FAP substrates may have potential as biomarkers of disease progression. Proteomics-based assays utilising selected reaction monitoring (SRM) can be designed and optimised to detect and quantify levels of peptides in complex biological samples. SRM analysis has previously been applied to not only determine levels of cleaved and full-length caspase substrates *in vivo*, but also the rate of cleavage [44]. Such techniques may be applicable to FAP substrates.

3 Targeting FAP

As the role of FAP seems to be highly contextual, it is important to consider the pathological situation in order to determine the best method to target FAP-expressing cells. A number of tools have therefore been designed to exploit the enzyme activity of FAP or to target this protein on cells.

3.1 Inhibitors

A major hurdle in the study of FAP enzyme activity has been the lack of selective inhibitors against this protease. FAP shares DPP specificity with the enzyme members of the DPP4 family, DPP4, DPP8 and DPP9, as well as endopeptidase specificity with prolyl endopeptidase (PREP). Thus, designing inhibitors that are selective for FAP over other DPPs and PREP is challenging. Recently, however, two independent groups have designed potent FAP-selective inhibitors [45, 46] (Table 1).

Poplawski et al. have recently developed potent FAP-selective and PREP-selective inhibitors using boronic acid-based compounds [46]. One compound, *N*-(pyridine-4-carbonyl)-D-Ala-boroPro, has a more than 350-fold selectivity for FAP over PREP, and also has very strong selectivity over DPP4, DPP8 and DPP9. The development of such a potent and selective inhibitor will no doubt prove extremely useful in the investigation of the biological role of FAP.

An independent study has developed a new class of FAP inhibitors based on an *N*-(4-quinolinoyl)-Gly-(2-cyanopyrrolidine) scaffold [45]. Of the 34 compounds

Table 1. Properties of potent FAP-selective inhibitors

Compound	IC ₅₀ (nM)					K _i (nM)		References
	FAP	DPP4	DPP8	DPP9	PREP	FAP	PREP	
<i>N</i> -(Pyridine-4-carbonyl)-D-Ala-boroPro, (compound 6)	36 ± 4.8	>100 000	5600 ± 1300	3400 ± 800	13 000 ± 4300	9 ± 0.9	3100 ± 260	[46]
Compound 7	10.3 ± 0.4	>100 000	N/A	>100 000	860 ± 70	3 ± 0.4	N/A	[45]

Values are expressed as ±SEM. N/A, not available.

developed in that study, compound 7 has particular selectivity towards FAP over the related proteases DPP4, DPP8 and DPP9, and is also selective over PREP. Modifications to the quinolinoyl ring improved the selectivity for FAP over PREP [45], indicating that further developments of this scaffold can yield compounds with more selectivity for FAP.

As combined inhibition of FAP and DPP4 enzyme activities attenuates tumour growth more than DPP4 inhibition alone [34, 47], a potent FAP-selective inhibitor may have potential for treating certain epithelial tumours and may be useful in a number of other clinical settings, such as atherosclerosis and arthritis [33]. The development of potent selective FAP inhibitors will be very useful in developing an understanding of the biological roles of FAP.

3.2 Prodrugs

Prodrugs consist of a cytotoxic agent coupled to a peptide that encodes the consensus sequence for cleavage by a particular protease. The cytotoxic agent becomes active upon release from the peptide, which only occurs in the presence of the specific protease (Fig. 4). Due to the restricted expression pattern and unique enzyme activity of FAP, FAP-activated prodrugs have potential as a therapeutic intervention in a number of pathological processes where FAP-expressing cells are implicated. Several FAP-activated prodrugs and protoxins have been designed.

The honeybee toxin melittin has been modified to produce an FAP-activated protoxin that causes lysis of FAP-positive tumour stromal cells [48]. Intra-tumoural injection of this protoxin into breast and prostate cancer xenographs in mice resulted in significant cell lysis and inhibition of tumour growth. However, intravenous administration of this protoxin caused haemolysis and death, indicating that use of this protoxin should be limited to situations where intra-tumoural injection is possible. Despite its limitations, this study demonstrates that FAP-activated protoxins can significantly impair tumour growth.

Doxorubicin has had limited clinical success as a chemotherapeutic agent due to cytotoxic side effects [49]. An FAP-activated doxorubicin prodrug has been shown to minimise these off-target effects [9]. This prodrug produced

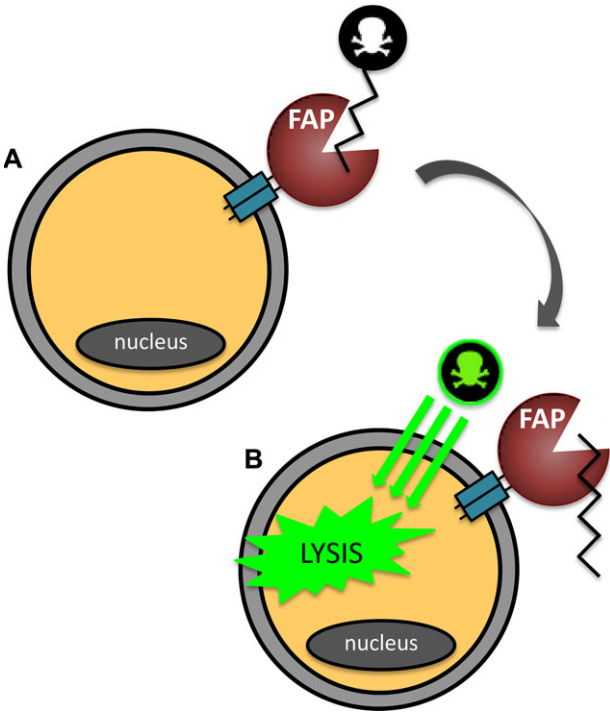


Figure 4. FAP-activated prodrugs. Prodrugs consist of a cytotoxic agent conjugated to a peptide sequence specific for a particular protease, in this case FAP. Exposure of the prodrug to FAP (A) results in cleavage of the peptide sequence thereby releasing and activating the cytotoxic agent (B), which acts on the cell.

similar anti-tumour effects to doxorubicin alone, without the severe cytotoxic side effects in normal tissue. However, low levels of non-specific activation of the prodrug were observed in normal tissue and plasma, which was attributed to the instability of the peptide bond linking the dipeptide to doxorubicin. However, the dipeptide sequence used in that study is known to be cleaved by other oligopeptidases, including PREP [8], which is ubiquitous in normal tissues. Such issues of linker instability, peptide specificity and protease distribution are commonly encountered in the development and use of protease-activated prodrugs.

Another FAP-activated prodrug contains the plant-derived toxin thapsigargin (TG) [50], which inhibits calcium pumps, leading to an intracellular build up of calcium, resulting in

cell death. The FAP-activated TG prodrug caused significant apoptosis of stromal cells, compared to untreated controls and mice treated with TG prodrugs conjugated to a scrambled peptide sequence. Although cell death was not observed in the cancer cells themselves, the stromal cell death was significant enough to inhibit growth of both human breast and human prostate cancer xenografts in that study [50].

3.3 T-cell immunotherapy

Based on its consistent expression in tumour stroma, FAP has become the focus of two recent studies utilising T-cell immunotherapy [51, 52]. In both of these studies, chimeric antigen receptors (CARs) reactive against FAP were genetically engineered and expressed by T cells.

Tran et al. looked at the effect of FAP-CAR T cells in mouse models of cancer as well as human pancreatic cancer xenografts [52]. Despite the *in vitro* efficacy of this FAP-CAR T-cell against recombinant FAP as well as HEK293 cells stably expressing FAP, a limited anti-tumour effect was observed in mouse cancer models. Unexpected off-target effects in bone marrow and skeletal muscle, including cachexia, were also observed. To further investigate this result, non-tumour-bearing mice were treated with FAP-CAR T cells but bone toxicities and cachexia were still observed. That study identified FAP-positive mesenchymal stromal cells in bone, adipose and muscle tissues in both non-tumour-bearing and tumour-bearing mice. Interestingly, similar side effects have been observed in a separate study in which FAP gene transcribing cells were targeted and killed with diphtheria toxin [53].

Kakarla et al. studied the effects of FAP-CAR T cells in a tumour initiation model as well as an established lung cancer xenograft model [51]. In both of these models, a significant decrease in FAP-positive stromal cells was observed as well as suppression of tumour growth. Although this anti-tumour effect was short lived, combined administration of FAP-CAR T cells and cancer cell targeted T cells led to a significant and prolonged inhibition of tumour growth. Furthermore, no off-target effects, including bone marrow abnormalities, were observed.

CAR T-cell immunotherapy relies on T-cell receptor specificity and avidity, so these disparate results may be explained following close examination of the CARs that were used. In addition, cytotoxic T cells are associated with cytokine release that may damage non-target cells. Parallel studies in FAP gene knockout mice would greatly assist in investigating the specificity of these FAP-CAR T cells and their effects *in vivo*.

3.4 Considerations when targeting FAP

Clearly there are significant differences between targeting FAP gene transcribing cells versus targeting FAP protein

or enzyme activity. Prodrugs exploit FAP enzyme activity to target and kill FAP-positive cells, whereas T-cell immunotherapy approaches target antigenic FAP protein to achieve this. Inhibitors, on the other hand, only block enzyme activity, leaving the cell intact. These are important considerations when determining which approach to use when targeting FAP, and also when interpreting the results from applying such techniques.

The non-concordant data obtained when targeting FAP-positive cells also raise questions about whether the expression patterns of FAP mRNA, protein and enzyme activity differ in normal adult tissues. The data might also be influenced by a natural FAP inhibitor, if it exists. Such an inhibitor may limit prodrug activation or compete with FAP inhibitory compounds, but investigation on this topic is needed.

A major limitation of antibody-based therapies is the limited availability of FAP-specific antibodies, particularly antibodies that cross-react with FAP derived from different species. This issue has been discussed thoroughly [19], and has hindered the implementation of antibody-based therapies in the clinical setting [54, 55]. This issue also highlights the advantages of targeting FAP enzyme activity with prodrugs and inhibitors, as the enzyme activity of this protease is conserved across species.

There clearly are large gaps in knowledge of the fundamental biology of this protease. The expression pattern and substrate repertoire of this protease require greater definition in order to elucidate the physiological roles of FAP to better target this protease in pathogenic settings. Improved knowledge of the basic biology of FAP will better predict potential effects of targeting this protease and also predict potential impacts on the proteome when FAP enzyme activity is inhibited or ablated.

4 Proteomic and degradomic applications

To date, most efforts towards understanding the biological functions of FAP have focussed on defining its cleavage site specificity [4, 7, 10]. These results are invaluable and have no doubt enabled the design and development of FAP-selective inhibitors and FAP-activated prodrugs. However, research into the degradome of this protease now needs to be undertaken.

Very few physiological substrates of FAP have been identified. Recently, new natural substrates were identified by screening known DPP4 substrates against FAP [11]. The four neuropeptides identified require *in vivo* investigation to determine the contribution of FAP to their physiological processing. This limited knowledge of the substrate repertoire of this protease emphasises the need to move away from traditional biochemical approaches and towards more unbiased, global approaches to identify novel physiological substrates of FAP.

A number of degradomic techniques have been developed for natural substrate discovery [56, 57]. Terminal amine isotopic labelling of substrates (TAILS) is a proven degradomic technique that identifies N-terminal cleavage products generated by a protease of interest [58]. The advantage of such global degradomic approaches, compared to traditional biochemical methods for substrate discovery, is that substrates exist in their native forms and locations [56]. The physiological setting of whether the substrate and protease actually come into contact is thereby taken into account. TAILS has been applied to a number of intracellular and extracellular proteases to successfully identify their substrates [59–62], including other members of the DPP4 family [63].

4.1 Proteomic and degradomic analyses of related proteases

A number of physiological DPP4, DPP8 and DPP9 substrates have been identified using proteomic- and degradomic-based techniques.

Tinoco et al. utilised a label-free LC-MS-based peptidomics platform to investigate the contribution of DPP4 enzyme activity to peptide metabolism in the kidney [64]. Global comparison of peptide levels in kidneys isolated from wild type and DPP4 gene knockout mice identified 70 renal substrates of DPP4. These substrates were validated *in vitro* using recombinant DPP4. It was also shown that aminopeptidases work in concert with DPP4 to catabolise Pro-containing peptides in the kidney. This method proved highly successful in implicating DPP4 in catabolic pathways in the kidney. However, a major contributing factor to this success was the ability to optimise the technique based on detection of known DPP4 substrates [65].

Unlike DPP4, the substrate repertoires of DPP8, DPP9 and FAP are poorly defined and therefore require more global, unbiased degradomic techniques.

Wilson et al. applied TAILS to identify 14 potential natural substrates of DPP8 and DPP9 in cytoplasmic extracts from SKOV3 cells overexpressing catalytically active or inactive human DPP8 or DPP9 [63]. Of these 14 natural substrates, nine were validated using peptide fragments *in vitro*. Further investigations will include whether naturally occurring forms of the putative substrates are hydrolysed.

4.2 Validation of substrates

A major hurdle in the application of degradomic techniques is refining the methods to validate and explore the biological significance of candidate substrates both *in vitro* and *in vivo*. There are multiple levels of validation, which all contribute to assigning a potential molecule as a physiological substrate of a protease.

Positional degradomic approaches, such as TAILS, are advantageous as they enable both identification of the sub-

strate as well as the site of cleavage. Thus, a lot of information can be drawn from such experiments, including cleavage site specificity. This provides an extra level of confidence if the cleavage site specificity of a protease is known and can therefore be matched to the observed cleavage site in the candidate substrate. In the case of FAP, cleavage occurs predominantly after a Pro residue, usually preceded by a Gly residue. TAILS data can therefore be manually parsed to identify cleavage sites occurring after Gly-Pro motifs.

Biochemical validation of candidate substrates can be performed through a variety of methods usually involving incubation of purified protease with purified substrate or peptides derived from the substrate spanning the cleavage site, and cleavage can be monitored via SDS-PAGE or MALDI-TOF-MS [58, 63, 66]. In addition, enrichment of full-length and cleaved forms of potential substrates can be performed using SRM or co-immunoprecipitation with antibodies specific to the mature- and neo-N-termini [66]. This can give an indication of cleavage events within the cellular environment and can also provide information on the contribution of cleavage by the protease of interest *in vitro*.

Jefferson et al. present a clear study of the levels of validation required to thoroughly confirm candidate substrates identified through degradomic approaches, as physiological substrates *in vivo* [61]. Degradomic analyses were performed to identify the cleavage site specificity and substrate repertoire for the astacin metalloprotease meprin β [61, 62]. A number of candidate substrates were identified, including the Alzheimer's disease-related protein amyloid precursor protein (APP). Initial validation studies were performed *in vitro* and *in cellulo*, which confirmed cleavage of APP by meprin β . *In vivo* analysis of mouse brains isolated from wild type and meprin β gene knockout mice revealed decreased processing of APP in the absence of meprin β [61]. Thus, it was confirmed that meprin β contributes to the physiological processing of APP. With the availability of the FAP gene knockout mouse, it is possible to apply a similar approach to FAP candidate substrates identified in future degradomic studies of FAP.

5 Conclusions

Being a protease, the fundamental role of FAP is to specifically process other proteins and peptides. Irreversible modification of substrates by proteases significantly alters their function, converting them from active to inactive proteins, or vice versa, releasing them from membranes via shedding or fragmenting them to form separate bioactive peptides [67]. In order to delineate the role of a protease and its impact on the proteome and cell biology, its substrate repertoire needs to be defined.

Recent approaches targeting FAP through the use of prodrugs, protoxins, inhibitors, antibodies and T cells have proved valuable in demonstrating the consistent expression of this protease in tumourigenesis and other pathological processes. However, the role of FAP seems to be very

contextual, with its expression and/or enzyme activity being detrimental in some pathological conditions whilst being beneficial in others. In order to determine the impact of ablating FAP-positive cells or inhibiting FAP enzyme activity, more focus needs to be placed on understanding the fundamental biology of this protease. Defining the substrate repertoire of FAP through the application of proteomic and degradomic analyses will greatly enhance our knowledge of FAP and its role in both physiological and pathological processes.

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6 References

- [1] Gorrell, M. D., Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin. Sci.* 2005, **108**, 277–292.
- [2] Zhang, H., Chen, Y., Keane, F. M., Gorrell, M. D., Advances in understanding the expression and function of dipeptidyl peptidase 8 and 9. *Mol. Cancer Res.* 2013, **11**, 1487–1496.
- [3] Gorrell, M. D., Park, J. E., in: Rawlings, N. L., Salvesen, G. (Eds.), *Handbook of Proteolytic Enzymes*, 3rd edition, Elsevier, San Diego 2013, pp. 3395–3401.
- [4] Aertgeerts, K., Levin, I., Shi, L., Snell, G. P. et al., Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein Alpha. *J. Biol. Chem.* 2005, **280**, 19441–19444.
- [5] Lee, K. N., Jackson, K. W., Terzyan, S., Christiansen, V. J., McKee, P. A., Using substrate specificity of antiplasmin-cleaving enzyme for fibroblast activation protein inhibitor design. *Biochemistry* 2009, **48**, 5149–5158.
- [6] Edosada, C. Y., Quan, C., Wiesmann, C., Tran, T. et al., Selective inhibition of fibroblast activation protein protease based on dipeptide substrate specificity. *J. Biol. Chem.* 2006, **281**, 7437–7444.
- [7] Edosada, C. Y., Quan, C., Tran, T., Pham, V. et al., Peptide substrate profiling defines fibroblast activation protein as an endopeptidase of strict Gly(2)-Pro(1)-cleaving specificity. *FEBS Lett.* 2006, **580**, 1581–1586.
- [8] Jambunathan, K., Watson, D. S., Endsley, A. N., Kodukula, K., Galande, A. K., Comparative analysis of the substrate preferences of two post-proline cleaving endopeptidases, prolyl oligopeptidase and fibroblast activation protein alpha. *FEBS Lett.* 2012, **586**, 2507–2512.
- [9] Huang, S., Fang, R., Xu, J., Qui, S. et al., Evaluation of the tumor targeting of a FAPalpha-based doxorubicin prodrug. *J. Drug Target.* 2011, **19**, 487–496.
- [10] Aggarwal, S., Brennen, W. N., Kole, T. P., Schneider, E. et al., Fibroblast activation protein peptide substrates identified from human collagen I derived gelatin cleavage sites. *Biochemistry* 2008, **47**, 1076–1086.
- [11] Keane, F. M., Nadvi, N. A., Yao, T.-W., Gorrell, M. D., Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY are novel substrates of fibroblast activation protein- α . *FEBS J.* 2011, **278**, 1316–1332.
- [12] Park, J. E., Lenter, M. C., Zimmermann, R. N., Garin-Chesa, P. et al., Fibroblast activation protein: a dual-specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J. Biol. Chem.* 1999, **274**, 36505–36512.
- [13] Levy, M. T., McCaughan, G. W., Abbott, C. A., Park, J. E. et al., Fibroblast activation protein: a cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodelling interface in human cirrhosis. *Hepatology* 1999, **29**, 1768–1778.
- [14] Lee, K. N., Jackson, K. W., Christiansen, V. J., Chung, K. H., McKee, P. A., A novel plasma proteinase potentiates alpha2-antiplasmin inhibition of fibrin digestion. *Blood* 2004, **103**, 3783–3788.
- [15] Lee, K. N., Jackson, K. W., Christiansen, V. J., Dolence, E. K., McKee, P. A., Enhancement of fibrinolysis by inhibiting enzymatic cleavage of precursor alpha2-antiplasmin. *J. Thromb. Haemost.* 2011, **9**, 987–996.
- [16] Frantz, C., Stewart, K. M., Weaver, V. M., The extracellular matrix at a glance. *J. Cell Sci.* 2010, **123**, 4195–4200.
- [17] Christiansen, V. J., Jackson, K. W., Lee, K. N., McKee, P. A., Effect of fibroblast activation protein and alpha2-antiplasmin cleaving enzyme on collagen types I, III, and IV. *Arch. Biochem. Biophys.* 2007, **457**, 177–186.
- [18] Lee, K. N., Jackson, K. W., Christiansen, V. J., Lee, C. S. et al., Antiplasmin-cleaving enzyme is a soluble form of fibroblast activation protein. *Blood* 2006, **107**, 1397–1404.
- [19] Jacob, M., Chang, L., Puré, E., Fibroblast activation protein in remodeling tissues. *Curr. Mol. Med.* 2012, **12**, 1220–1243.
- [20] Niedermeyer, J., Garin-Chesa, P., Kriz, M., Hilberg, F. et al., Expression of the fibroblast activation protein during mouse embryo development. *Int. J. Dev. Biol.* 2001, **45**, 445–447.
- [21] Brown, D. D., Wang, Z., Furlow, J. D., Kanamori, A. et al., The thyroid hormone-induced tail resorption program during *Xenopus laevis* metamorphosis. *Proc. Natl. Acad. Sci. USA* 1996, **93**, 1924–1929.
- [22] Niedermeyer, J., Kriz, M., Hilberg, F., Garin-Chesa, P. et al., Targeted disruption of mouse fibroblast activation protein. *Mol. Cell. Biol.* 2000, **20**, 1089–1094.
- [23] Niedermeyer, J., Scanlan, M. J., Garin-Chesa, P., Daiber, C. et al., Mouse fibroblast activation protein: molecular cloning, alternative splicing and expression in the reactive stroma of epithelial cancers. *Int. J. Cancer* 1997, **71**, 383–389.
- [24] Rettig, W. J., Garin-Chesa, P., Beresford, H. R., Oettegen, H. F. et al., Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. *Proc. Natl. Acad. Sci. USA* 1988, **85**, 3110–3114.
- [25] Bauer, S., Jendro, M. C., Wadle, A., Kleber, S. et al., Fibroblast activation protein is expressed by rheumatoid myofibroblast-like synoviocytes. *Arthritis Res. Ther.* 2006, **8**, R171.

- [26] Milner, J. M., Kevorkian, L., Young, D. A., Jones, D. et al., Fibroblast activation protein alpha is expressed by chondrocytes following a pro-inflammatory stimulus and is elevated in osteoarthritis. *Arthritis Res. Ther.* 2006, 8, R23.
- [27] Brokopp, C. E., Schoenauer, R., Richards, P., Bauer, S. et al., Fibroblast activation protein is induced by inflammation and degrades type I collagen in thin-cap fibroatheromata. *Eur. Heart J.* 2011, 32, 2713–2722.
- [28] Wang, X. M., Yu, D. M. T., McCaughan, G. W., Gorrell, M. D., Fibroblast activation protein increases apoptosis, cell adhesion and migration by the LX-2 human stellate cell line. *Hepatology* 2005, 42, 935–945.
- [29] Levy, M. T., McCaughan, G. W., Marinos, G., Gorrell, M. D., Intrahepatic expression of the hepatic stellate cell marker fibroblast activation protein correlates with the degree of fibrosis in hepatitis C virus infection. *Liver Internat.* 2002, 22, 93–101.
- [30] Cox, G., Kable, E., Jones, A., Fraser, I. et al., 3-dimensional imaging of collagen using second harmonic generation. *J. Struct. Biol.* 2003, 141, 53–62.
- [31] Garin-Chesa, P., Old, L. J., Rettig, W. J., Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc. Natl Acad. Sci. USA* 1990, 87, 7235–7239.
- [32] Christiansen, V. J., Jackson, K., Lee, K., Downs, T., McKee, P. A., Targeting inhibition of fibroblast activation protein-alpha and prolyl oligopeptidase activities on cells common to metastatic tumor microenvironments. *Neoplasia* 2013, 15, 348–358.
- [33] Keane, F. M., Chowdhury, S., Yao, T.-W., Nadvi, N. A. et al., in: Dunn, B. (Ed.), *Proteinases as Drug Targets*, Royal Society of Chemistry, Cambridge, UK 2012, pp. 119–145.
- [34] Santos, A. M., Jung, J., Aziz, N., Kissil, J. L., Pure, E., Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *J. Clin. Invest.* 2010, 119, 3613–3626.
- [35] Brennen, W. N., Isaacs, J. T., Denmeade, S. R., Rationale behind targeting fibroblast activation protein-expressing carcinoma-associated fibroblasts as a novel chemotherapeutic strategy. *Mol. Cancer Ther.* 2012, 11, 257–266.
- [36] Gao, M. Q., Kim, B. G., Kang, S., Choi, Y. P. et al., Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial-mesenchymal transition-like state in breast cancer cells in vitro. *J. Cell Sci.* 2010, 123, 3507–3514.
- [37] Kraman, M., Bambrough, P. J., Arnold, J. N., Roberts, E. W. et al., Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein- α . *Science* 2010, 330, 827–830.
- [38] Aimes, R. T., Zijlstra, A., Hooper, J. D., Ogbourne, S. M. et al., Endothelial cell serine proteases expressed during vascular morphogenesis and angiogenesis. *Thromb. Haemost.* 2003, 89, 561–572.
- [39] Goodman, J., Rozypal, T., Kelly, T., Seprase, a membrane-bound protease, alleviates the serum growth requirement of human breast cancer cells. *Clin. Exp. Metastasis* 2003, 20, 459–470.
- [40] Zukowska, Z., Pons, J., Lee, E. W., Li, L., Neuropeptide Y: a new mediator linking sympathetic nerves, blood vessels and immune system? *Can. J. Physiol. Pharmacol.* 2003, 81, 89–94.
- [41] Cohen, S. J., Alpaugh, R. K., Palazzo, I., Meropol, N. J. et al., Fibroblast activation protein and its relationship to clinical outcome in pancreatic adenocarcinoma. *Pancreas* 2008, 37, 154–158.
- [42] Cheng, J. D., Dunbrack, R. L., Valianou, M., Rogatko, A. et al., Promotion of tumour growth by murine fibroblast activation protein, a serine protease, in an animal model. *Cancer Res.* 2002, 62, 4767–4772.
- [43] Keane, F., Yao, T.-W., Seelk, S., Gall, M. G. et al., Quantitation of fibroblast activation protein (FAP)-specific protease activity in mouse, baboon and human fluids and organs using novel substrate 3144-AMC. *FEBS Open Bio.* 2014, 4, 43–54.
- [44] Agard, N. J., Mahrus, S., Trinidad, J. C., Lynn, A. et al., Global kinetic analysis of proteolysis via quantitative targeted proteomics. *Proc. Natl Acad. Sci. USA* 2012, 109, 1913–1918.
- [45] Jansen, K., Heirbaut, L., Cheng, J. D., Joossens, J. et al., Selective inhibitors of fibroblast activation protein (FAP) with a (4-Quinolinyloxy)-glycyl-2-cyanopyrrolidine scaffold. *ACS Med. Chem. Lett.* 2013, 4, 491–496.
- [46] Poplawski, S. E., Lai, J. H., Li, Y., Jin, Z. et al., Identification of selective and potent inhibitors of fibroblast activation protein and prolyl oligopeptidase. *J. Med. Chem.* 2013, 56, 3467–3477.
- [47] Walsh, M. P., Duncan, B., Larabee, S., Krauss, A. et al., Val-BoroPro accelerates T cell priming via modulation of dendritic cell trafficking resulting in complete regression of established murine tumors. *PLoS ONE* 2013, 8, e58860.
- [48] LeBeau, A. M., Brennen, W. N., Aggarwal, S., Denmeade, S. R., Targeting the cancer stroma with a fibroblast activation protein-activated promelittin protoxin. *Mol. Cancer Ther.* 2009, 8, 1378–1386.
- [49] Chen, Y., Hu, L., Design of anticancer prodrugs for reductive activation. *Med. Res. Rev.* 2008, 29, 29–64.
- [50] Brennen, W. N., Rosen, D. M., Wang, H., Isaacs, J. T., Denmeade, S. R., Targeting carcinoma-associated fibroblasts within the tumour stroma with a fibroblast activation protein-activated prodrug. *J. Natl. Cancer Inst.* 2012, 104, 1320–1334.
- [51] Kakarla, S., Chow, K. K. H., Mata, M., Schaffer, D. R. et al., Antitumour effects of chimeric receptor engineered human T cells directed to tumour stroma. *Molec. Ther.* 2013, 21, 1611–1620.
- [52] Tran, E., Chinnasamy, D., Yu, Z., Morgan, R. A. et al., Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia. *J. Exp. Med.* 2013, 210, 1125–1135.
- [53] Roberts, E. W., Deonaraine, A., Jones, J. O., Denton, A. E. et al., Depletion of stromal cells expressing fibroblast activation protein- α from skeletal muscle and bone marrow results in cachexia and anemia. *J. Exp. Med.* 2013, 210, 1137–1151.
- [54] Hofheinz, R., Al-Batran, S., Hartmann, F., Hartung, G. et al., Stromal antigen targeting by a humanised monoclonal

- antibody: an early phase II trial of sibtuzumab in patients with metastatic colorectal cancer. *Onkologie* 2003, 26, 44–48.
- [55] Scott, A. M., Wiseman, G., Welt, S., Adjei, A. et al., A phase I dose-escalation study of sibtuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. *Clin. Cancer Res.* 2003, 9, 1639–1647.
- [56] Overall, C. M., Blobel, C. P., In search of partners: linking extracellular proteases to substrates. *Nature Rev. Mol. Cell Biol.* 2007, 8, 245–257.
- [57] López-Otin, C., Overall, C. M., Protease degradomics: a new challenge for proteomics. *Nat. Rev. Mol. Cell Biol.* 2002, 3, 509–519.
- [58] Kleifeld, O., Doucet, A., Keller, U. a. d., Prudova, A. et al., Isotopic labeling of terminal amines in complex samples identifies protein N-termini and protease cleavage products. *Nat. Biotechnol.* 2010, 28, 281–291.
- [59] Prudova, A., auf dem Keller, U., Butler, G. S., Overall, C. M., Multiplex N-terminome analysis of MMP-2 and MMP-9 substrate degradomes by iTRAQ-TAILS quantitative proteomics. *Mol. Cell. Proteomics* 2010, 9, 894–911.
- [60] Tholen, S., Biniossek, M. L., Gessler, A., Mueller, S. et al., Contribution of cathepsin L to secretome composition and cleavage pattern of mouse embryonic fibroblasts. *Biol. Chem.* 2011, 392, 961–971.
- [61] Jefferson, T., Causevic, M., Keller, U. a. d., Schilling, O. et al., Metalloprotease meprin beta generates nontoxic N-terminal amyloid precursor protein fragments in vivo. *J. Biol. Chem.* 2011, 286, 27741–27750.
- [62] Becker-Pauly, C., Barre, O., Schilling, O., auf dem Keller, U. et al., Proteomic analyses reveal an acidic prime side specificity for the astacin metalloprotease family reflected by physiological substrates. *Mol. Cell. Proteomics* 2011, 10, 1–19.
- [63] Wilson, C. H., Inadarto, D., Doucet, A., Pogson, L. D. et al., Identifying natural substrates for dipeptidyl peptidases 8 and 9 using terminal amine isotopic labeling of substrates (TAILS) reveals in vivo roles in cellular homeostasis and energy metabolism. *J. Biol. Chem.* 2013, 288, 13936–13949.
- [64] Tinoco, A. D., Tagore, D. M., Saghatelian, A., Expanding the dipeptidyl peptidase 4-regulated peptidome via an optimized peptidomics platform. *J. Am. Chem. Soc.* 2010, 132, 3819–3830.
- [65] Tagore, D. M., Nolte, W., Neveu, J., Rangel, R. et al., Peptidase substrates via global peptide profiling. *Nat. Chem. Biol.* 2009, 5, 23–25.
- [66] Starr, A. E., Overall, C. M., in: Handel, T. M., Hamel, D. L. (Eds), *Methods in Enzymology*, Academic Press, New York, 2009, pp. 281–307.
- [67] Doucet, A., Butler, G. S., Rodriguez, D., Prudova, A., Overall, C. M., Metadegradomics. *Mol. Cell. Proteomics* 2008, 7, 1925–1951.
- [68] Yu, D. M. T., Yao, T.-W., Chowdhury, S., Nadvi, N. A. et al., The dipeptidyl peptidase IV family in cancer and cell biology. *FEBS J.* 2010, 277, 1126–1144.