What doesn't kill you makes you stranger: Dipeptidyl peptidase-4 (CD26) proteolysis differentially modulates the activity of many peptide hormones and cytokines generating novel cryptic bioactive ligands

Ahmed M. Elmansi [a,b], Mohamed E. Awad [c], Nada H. Eisa [d,e], Dmitry Kondrikov [a,b], Khaled A. Hussein [f], Alexandra Aguilar-Pérez [g,h,i], Samuel Herberg [j], Sudharsan Periyasamy-Thandavan [d], Sadanand Fulzele [k,l], Mark W. Hamrick [i,k,l], Meghan E. McGee-Lawrence [i,k,l], Carlos M. Isales [k,l,m], Brian F. Volkman [n], William D. Hill [a,b,i,k,l],*

[a] Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29403, United States
[b] Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29403, United States
[c] Department of Oral Biology, School of Dentistry, Augusta University, Augusta, GA 30912, United States
[d] Georgia Cancer Center, Augusta University, Augusta, GA 30912, United States
[e] Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt
[f] Department of Surgery and Medicine, National Research Centre, Cairo, Egypt
[g] Department of Anatomy and Cell Biology, Indiana University School of Medicine in Indianapolis, IN, United States
[h] Department of Cellular and Molecular Biology, School of Medicine, Universidad Central del Caribe, Bayamon, 00956, Puerto Rico
[i] Cellular Biology and Anatomy, Medical College of Georgia, Augusta University, Augusta, GA 30912, United States
[j] Departments of Ophthalmology & Cell and Dev. Bio., SUNY Upstate Medical University, Syracuse, NY 13210, United States
[k] Department of Orthopaedic Surgery, Medical College of Georgia, Augusta University, Augusta, GA 30912, United States
[l] Center for Healthy Aging, Medical College of Georgia, Augusta University, Augusta, GA, 30912, United States
[m] Division of Endocrinology, Diabetes and Metabolism, Medical College of Georgia, Augusta University, Augusta, GA 30912, United States
[n] Biochemistry Department, Medical College of Wisconsin, Milwaukee, WI 53226, United States

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ABSTRACT

Dipeptidyl peptidase 4 (DPP4) is an exopeptidase found either on cell surfaces where it is highly regulated in terms of its expression and surface availability (CD26) or in a free/circulating soluble constitutively available and intrinsically active form. It is responsible for proteolytic cleavage of many peptide substrates. In this review we discuss the idea that DPP4-cleaved peptides are not necessarily inactivated, but rather can possess either a modified receptor selectivity, modified bioactivity, new antagonistic activity, or even a novel activity relative to the intact parent ligand.

We examine in detail five different major DPP4 substrates: glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide tyrosine-tyrosine (PYY), and neuropeptide Y (NPY), and stromal derived factor 1 (SDF-1 aka CXCL12). We note that discussion of the cleaved forms of these five peptides are underrepresented in the research literature, and are both poorly investigated and poorly understood, representing a serious research literature gap. We believe they are understudied and misinterpreted as inactive due to several factors. This includes lack of accurate and specific quantification methods, sample collection techniques that are inherently inaccurate and inappropriate, and a general perception that DPP4 cleavage inactivates its ligand substrates.

Increasing evidence points towards many DPP4-cleaved ligands having their own bioactivity. For example, GLP-1 can work through a different receptor than GLP-1R, DPP4-cleaved GIP can function as a GIP receptor antagonist at high doses, and DPP4-cleaved PYY, NPY, and CXCL12 can have different receptor selectivity, or can bind novel, previously unrecognized receptors to their intact ligands, resulting in altered signaling and functionality. We

believe that more rigorous research in this area could lead to a better understanding of DPP4's role and the biological importance of the generation of novel cryptic ligands. This will also significantly impact our understanding of the clinical effects and side effects of DPP4-inhibitors as a class of anti-diabetic drugs that potentially have an expanding clinical relevance. This will be specifically relevant in targeting DPP4 substrate ligands involved in a variety of other major clinical acute and chronic injury/disease areas including inflammation, immunology, cardiology, stroke, musculoskeletal disease and injury, as well as cancer biology and tissue maintenance in aging.

Contents

1. Introduction

Dipeptidyl peptidase 4 (DPP4) is a highly conserved exopeptidase found widely on the surface of epithelial, endothelial, stromal, stem and immune cells (Mulvihill & Drucker, 2014). In its cell membrane associated form it is referred to as cluster of differentiation 26 (CD26). DPP4 is a ubiquitous enzyme that is expressed in different compartments, including liver, kidney, gastrointestinal tract (GIT) and bone marrow (Ambrosi et al., 2017; Mentlein, 1999; Ou, O'Leary, & Broxmeyer, 2013). It also has a soluble form (sCD26/DPP4) that can be found in the circulation and in various tissue compartments. DPP4 regulates the activity of many different chemokines and peptide hormones, and it acts by cleaving off N-terminus dipeptides typically with a penultimate alanine or proline, although under some conditions other amino acids may be targeted (Broxmeyer, 2013; Drucker, 2007; Mortier, Gouwy, Van Damme, Proost, & Struyf, 2016).

CD26 is found as a single pass transmembrane protein that forms homodimers on most cells and in the majority of tissues (Mulvihill & Drucker, 2014). Regulation of trans-membrane CD26 activity occurs at multiple levels, including control of gene and protein expression, regulation of the transfer of CD26 to the cell surface, interaction with binding partners, and modulation of enzyme activity (Mulvihill & Drucker, 2014). Expression of cell surface trans-membrane, as well as intercellular membrane pools of trans-membrane CD26, is tightly controlled. Transition of CD26 to the surface of cells is highly regulated and can be initiated rapidly resulting in cell surface levels going from no CD26, or minimal basal levels, to very high levels in response to a large number of cell and tissue targeting factors such as interleukines, hypoxia-inducible factor 1 (HIF-1), interferons, retinoic acid, hepatocyte nuclear factor (HNF), granulocyte-colony stimulating factor (G-CSF), angiotensinogen II, classical leukocyte cellular activation (e.g. of T and B Cells), as well as modification of activity by post-translational glycosylation and subunit dimerization (Aroor et al., 2016; Bauvois, Djavaheri-Mergny, Rouillard, Dumont, & Wietzerbin, 2000; Christopherson, Cooper, & Broxmeyer, 2003; Chung et al., 2010; Dang et al., 2008; De Meester, Korom, Van Damme, & Scharpe, 1999; Gu et al., 2008; Klemann, Wagner, Stephan, & von Hörsten, 2016; Metzemaekers, Van Damme, Mortier, & Proost, 2016; Mortier et al., 2016; Mulvihill & Drucker, 2014; Shin et al., 2017). Functionally, DPP4 is involved in numerous cellular processes including cell-cell interaction, cell mobilization, cell migration, and membrane-associated activation of intracellular signal transduction pathways (De Meester et al., 1999; Lambeir, Durinx, Scharpé, & De Meester, 2003). CD26 is a relatively large protein consisting of 766 amino acids, most of which are extracellular, with a hydrophobic transmembrane anchor from amino acids 7 to 28, and a very short intracellular sequence consisting of 6 amino acids (Abbott, Baker, Sutherland, & McCaughan, 1994). The soluble form of the peptidase (sCD26/DPP4), composed of the extracellular amino acids 39-766, is shed into the extracellular environment by a number of matrix metalloproteinases (Havre et al., 2008; Varin et al., 2018). Unlike the tightly regulated expression/activity of the transmembrane parent form (CD26) on cells and within tissues, the soluble DPP4 (sCD26) is widely present in plasma and other tissue compartments and is constitutively fully enzymatically active (Klemann et al., 2016; Mortier et al., 2016). Indeed endogenous levels of soluble DPP4 may not represent a rate-limiting factor, allowing cleavage of most of its substrate pools in those compartments (Carbone, Buzkova, Fink, Robbins, Bethel, Hamrick, et al., 2017; Carbone, Buzkova, Fink, Robbins, Bethel, Isales, et al., 2017).

Only a limited number of review and primary experimental papers have highlighted the complex role that DPP4 plays in the generation of cryptic substrate ligands and the need to consider how these changes to the nature of the truncated substrate's signaling and functional activity impacts experimental data interpretation (Mortier, Van Damme, & Proost, 2008; Mulvihill & Drucker, 2014; O'Leary, Ou, & Broxmeyer, 2013). As a consequence, the idea that DPP4 proteolysis leads to partial or total inactivation of its substrates is still widely accepted and taken for granted. However, there is a growing literature that supports the idea that DPP4 metabolites can have selective, antagonistic or even novel signaling properties and functions (Cheng, Eby, LaPorte, Volkman, & Majetschak, 2017; Janssens et al., 2017; Kim, Yu, & Lee, 2014; Klemann et al., 2016; Mentlein, 1999; Mulvihill & Drucker, 2014; Ou et al., 2013; Szpakowska et al., 2018; Tarantola et al., 2012; Ziarek et al., 2017). We believe that the concept that in many cases DPP4 can alter substrate function, rather than just inactivating the ligand's functionality is still an underrepresented view in the overall scientific literature; one that carries tremendous experimental, interpretative as well as clinical repercussions. In this review, we highlight five major DPP4 substrates that are not inactivated, but rather demonstrate new, or revealed cryptic, interactions and function. We

show that DPP4 can modulate the function of the substrate not only by interfering with the substrate's ability to either bind or activate its receptor, but truncation can antagonize the receptor or alter the ratio of binding and activation between primary and secondary receptors or lead to interactions with a new receptor by the cleaved substrate that is not a target of the intact parent ligand. In many cases, DPP4-truncated peptides have complex novel functions that are not adequately appreciated. Excitingly, understanding this unexpected cryptic bioactivity is an important new area for research as it is underappreciated and represents a major gap in the research literature. The cryptic ligand activities come with significant clinical implications and call for new interpretations of some previous research results (Metzemaekers et al., 2016; Mulvihill & Drucker, 2014; Ou et al., 2013).

This review will focus on DPP4 substrates that have bioactivity upon cleavage by DDP4. We are referring to these substrates in the title as cryptic bioactive ligands, which means that they develop a new previously hidden, bioactivity after post-translational modification (Brandt, Lambeir, Maes, Scharpe, & De Meester, 2006). These substrates include glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide tyrosine-tyrosine (PYY), neuropeptide Y (NPY), and the cytokine Stromal Cell-Derived Factor-1 (SDF-1, or CXCL12 in the revised cytokine nomenclature (Zlotnik & Yoshie, 2000)). These five substrates can be classified into three main classes: incretin hormones, pancreatic polypeptides, and chemokines. Both GLP-1 and GIP are incretin peptides released by gut, and are mainly involved in postprandial activation of insulin secretion and clearance of glucose (Drucker, 2003). PYY and NPY are both members of the pancreatic polypeptide family, which is involved in regulating a large array of biological functions including appetite control, behavioral stress responses, angiogenesis, and blood pressure (Groneberg, Folkerts, Peiser, Chung, & Fischer, 2004; Mentlein, 1999). A subset of chemokines and cytokines are starting to be recognized as DPP4 targets that may have biological functions following cleavage (Broxmeyer, Capitano, Campbell, Hangoc, & Cooper, 2016; Metzemaekers et al., 2016; Ou et al., 2013; Vanheule, Metzemaekers, Janssens, Struyf, & Proost, 2018). CXCL12 is the best studied of these. CXCL12 is a chemokine/cytokine involved in regulation of cell mobilization, migration and homing during tissue development, tissue maintenance and disease for numerous cell types, as well as being involved in the regulation of complex processes such as angiogenesis, hematopoiesis, and tissue repair (Brandt et al., 2006; Bromage, Davidson, & Yellon, 2014). While we have selected these five specifically due to their relatively accepted and maturing presence in the literature - they are not the only examples of DPP4 substrates with cryptic bioactivity. Additionally, not all DPP4 substrate metabolites will likely have cryptic bioactivity. We will introduce each one of these five substrates individually and compare bioactivity of the intact ligand and the DPP4-cleaved ligand, based on recent research. We will also discuss why some results in this area have been misleading or hard to accurately interpret, and demonstrate what we believe are essential tools for better and more accurate experimental results in the future for this field.

Problems related to the lack of understanding of these cryptic ligands include: 1. There are few tools to distinguish the 'intact' ligand from the DPP4-cleaved ligand (or further proteolytically processed isoforms). Most antibodies to the ligands see both intact and N-terminal proteolytically cleaved isoforms equally well (Wang et al., 2014); 2. Consequently the short half-life, on the order of minutes, for most DPP4 ligand substrates leads to almost universal misinterpretation of the amount of intact ligands relative to the DPP4 cleaved ligands, which means some activities attributed to "intact" ligands are in fact due to the DPP4-cleaved isoforms; 3. Without the timely use of DPP4 inhibitors, or other proteolytic inhibitors, DPP4 activity continues during the time required to obtain and process the sample before stabilization and storage, with both *in vivo* and *in vitro* samples (e.g. blood draws followed by serum or plasma collection; or tissue culture media collection). This creates a number of problems when subsequently attempting to quantify ligands from such samples. For example, the actual amount of intact ligand is reduced by DPP4 activity, and once DPP4 initiates N-terminal cleavage, this allows other proteases to rapidly remove additional N-terminal amino acids accelerating the substrate's degradation and clearance (Klemann et al., 2016) leading to a significant reduction in the "total" amount of detectable ligand within a sample (Baerts et al., 2015; Richter et al., 2014). Complicating the problem of mismeasurement of "total" ligand isoforms is increased variability when DPP4 and other protease inhibitors are not used at the point of initial sample collection, a significantly increased portion of the remaining ligand isoforms collected are in the DPP4-cleaved state, or even further processed. This results in increased variability in sample ligand measurement, as well as variability in the identification and quantification of the different biologically active cleaved ligand isoforms (Baerts et al., 2015). These challenges will be discussed in more detail below.

In this review, we look at the effect that DPP4 proteolytic cleavage has on the activity and biological functions of five major DPP4 substrate peptide hormones. This review establishes a better understanding of the clinical relevance of DPP4 inhibitors, by focusing on the biological actions of the cleaved isoforms, which are significantly understudied and usually overlooked when compared to the biological actions of intact isoforms of different DPP4 substrates. We report the most recent findings in this field, and present evidence that DPP4-cleaved peptides are far more interesting, as well as translationally and clinically relevant, than initially thought.

2. DPP4

2.1. DPP4 activity

Membrane associated and soluble DPP4 enzymes are responsible for cleaving several circulating substrates including peptide hormones and cytokines. This functional cleaving of dipeptides occurs at the N-terminal of the target substrate, by specifically cleaving off X-Proline and X-Alanine dipeptides from its polypeptide substrates (Lone, Nolte, Tinoco, & Saghatelian, 2010; Mabilleau, Mieczkowska, & Chappard, 2013). Recently CD26/DPPIV is also recognized to cleave some substrates that include hydroxyproline, serine, glycine, valine, threonine, and leucine in the second amino acid position (Havre et al., 2008). CD26 forms homodimer complexes with the enzymatic activity residing in the extracellular portion and with each subunit containing an alpha/beta hydrolase domain together with an eight-bladed beta-propeller domain to form the substrate binding pocket (Rasmussen, Branner, Wiberg, & Wagtmann, 2003). DPP4 is a member of a complex gene family that contains other enzymes responsible for cleaving other structurally related peptides. The DPP4-related enzymes include seprase, fibroblast activation protein α, DPP-6, -8, and -9, Attractin, and N-acetylated-α-linked acidic dipeptidases I and II (Busek, Malik, & Sedo, 2004; Frerker et al., 2007; Gorrell, 2005). Over the last few years, DPP4 has been thoroughly investigated because of its impact on several crucial cytokines and peptides implicated in different medical conditions. One of the most studied DPP4 substrates is GLP-1. GLP-1 is a 30 amino acid long incretin peptide hormone essential for glucose metabolism. GLP-1 enhances insulin secretion, which makes its role critical in the physiology and pathophysiology of diabetes. Normally, GLP-1 gets cleaved rapidly by DPP4 (Cantini, Di Franco, Mannucci, & Luconi, 2017; Xu et al., 2007). GLP-1–based drugs, including GLP-1 agonists and DPP4 inhibitors have been recently introduced into clinical practice with much enthusiasm (Carr et al., 2010; Mannucci et al., 2005).

In addition to its dipeptidase enzymatic activity, CD26 is also reported to have non-enzymatic functions mediated by molecular interactions with multiple binding sites in the beta-propeller domain (Havre et al., 2008). CD26 can form heterodimers with fibroblast activation protein α, and to associate with plasminogen 2, adenosine deaminase (ADA), CD45, C-X-C chemokine receptor type 4 (CXCR4), and mannose 6-phosphate/insulin-like growth factor II receptor, as well as

binding to extracellular matrix (ECM) glycoproteins such as fibronectin and collagen proteins (Havre et al., 2008). DPP4 binding to ECM components may mediate turnover or modification of the ECM and DPP4 substrates near the cell surface. It permits soluble DPP4 to reside in specific ECM locations for either substrate interactions or DPP4 enzyme storage in the ECM reservoir for subsequent release from this pool, and attack of its substrates, upon ECM degradation during development, tissue maintenance or injury repair. A number of ECM molecules, including collagens and glycoproteins are modified by proteases to yield novel matrikines, which are ECM molecule fragments that act as trophic and growth factors. Proteolytic modification of ECM molecules can also yield novel binding sites for integrins and other EM receptors called matricryptins (Ricard-Blum & Salza, 2014; Su et al., 2016). If CD26 itself is not cleaving these ECM molecules directly it may be interacting with other proteases in regulating the creation of these molecules. The consequences of these interactions are unclear although they may serve to help rapidly modify ligands or binding partners of these molecules regulating signaling and adhesive interactions. Additionally, CD26 can internalize with some of these molecules, notably CD26 has been shown to bind CXCR4, the primary receptor for the cytokine/chemokine, and CD26 substrate, CXCL12 (Havre et al., 2008; Herrera et al., 2001). When CXCL12 binds CXCR4 initiating signaling, the CXCR4/CXCL12/CD26 complex can be internalized as part of the endosomal/lysosomal network. The exact function is not fully understood, but it may be part of the negative feedback regulation of the CXCL12/CXCR4 signaling pathway to temporally sharpen the end of the signal. The interaction of CD26 and CXCR4 will be further discussed in Section 7.

A naturally occurring loss of function mutation in the DPP4 gene helped identify the importance of the DPP4 enzyme. Fischer 344 (F344) rats have a Gly633-Arg mutation in the DPP4 gene. Although the mutant enzyme is synthesized normally, it is unable to be exported out of the endoplasmic reticulum, resulting in its rapid degradation. F344 rats showed a better glucose tolerance with higher GLP-1, GIP and insulin levels following an oral glucose challenge. The same results were demonstrated again in a 7-week high-fat feeding F344 rat model, in addition to reduced weight gain. Overall, it was shown that loss of DPP4 activity in rats is associated with an increase in GLP-1 action (Erickson, Suzuki, Sedlmayer, & Kim, 1992; Thompson et al., 1991; Tsuji et al., 1992).

DPP4 inhibitors have been intensely investigated gaining wide popularity as a therapeutic approach for diabetes. Until just few year ago, the majority of scientists believed that DPP4 inactivates peptide hormones by cleaving them. However, recent data showed that this is not always the case. A study by Ban et al., reported that DDP4-truncated peptide hormones may still retain functional activity similar to the non-truncated peptides (Ban et al., 2010). More interestingly, other studies demonstrated that the DPP4-truncated peptides have different receptor specificities leading to a modified activity different from the parent peptide (Gautier-Stein & Mithieux, 2013; Sparre-Ulrich et al., 2017). Importantly, emerging research suggests the activity of some peptides is mainly attributed to the cleaved form instead of the intact or non-cleaved parent form (Deacon, Plamboeck, Møller, & Holst, 2002). Understanding this crucial stem-point would explain the heterogeneity of the results of many, previously performed studies. This review shows it is common, yet inaccurate, to simply attribute the

experimental results to administrating the intact "non-cleaved" form of a DPP4 enzyme substrate. Rather, further investigation can reveal that the results are in fact due to a proteolytically modified form of the administered ligand substrate.

2.2. DPP4 substrates

The vast majority of research performed on DPP4 substrates is focused on the intact isoform of the substrate, as they are generally treated as being the sole bioactive form. This common assumption has resulted in a limited number of DPP4 substrates that have been well studied in terms of their proteolytic metabolic isoforms following DPP4 truncation, which is often the first proteolytic N-terminal step for DPP4 substrates (Klemann et al., 2016). Since DPP4 inhibitors used in the clinic are for treating diabetes, most DPP4-related research focuses on the incretin hormones GLP-1 or GIP as the target ligand in diabetic models.

We identified five major DPP4 substrates where research has focused on both their cleaved, as well as the intact, isoform (Table 1). Those ligand substrates include GLP-1, GIP, PYY, NPY, and CXCL12. Both GLP-1 and GIP are incretin hormones that are mainly involved in postprandial glucose level regulation (Nauck, 2013; Elahi, 1994). PYY and NPY belong to the polypeptide family of peptide hormones, and they both regulate appetite and satiety centers in brain (Flynn, Plata-Salamán, & Ffrench-Mullen, 1998; Unniappan et al., 2006). CXCL12 is a cytokine/chemokine and chemoattractant involved in regulating cell migration, homing of stem cells to injury sites, as well as the mobilization and recruiting of lymphocytes to sites of inflammation, or activated endothelium initiating cell attachment and diapedesis (Ghadge, Muhlstedt, Ozcelik, & Bader, 2011; Guo et al., 2015; Xue et al., 2014; Yu et al., 2016; Zhang et al., 2017). CXCL12 mediates the movement, differentiation and function of numerous other cells including various stem cells, neurons, angioblast/endothelial cells and tumor cells during tissue development, maintenance and disease, as well as complex processes such as angiogenesis, hematopoiesis, and tissue protection and repair following injury (e.g. cardioprotection, tissue response to ischeamic stroke, and bone repair) (Anderluh et al., 2016; Bromage et al., 2014; Cheng et al., 2017; Yang et al., 2018).

2.3. Troubleshooting the challenges in studying DPP4-cleaved substrates

While the listed DPP4 substrates are thoroughly studied in their intact form, more limited, but an increasing amount of research has been conducted to investigate dose effects, signaling pathways, and functions, related to their DPP4-cleaved forms. Furthermore, accurate interpretation of intact substrate research results is complicated by the common assumption by researchers that relates their outcome measures - any biological response or signaling event - to the intact form, without giving consideration to the idea that the cleaved form might at least partially be responsible for the observed effects.

One issue that complicates research of DPP4 metabolic substrates is the lack of commercially-available, reliable, sensitive, and accurate quantification methods to detect and measure the cleaved isoforms or even distinguish them from the intact substrates. Rigorous research requires the ability to accurately quantify both intact and DPP4-cleaved

Table 1
DPP4 intact substrates, and their amino acid sequences

Substrate	Amino acid sequence	Ref.
GLP-1(7-36)	HA ↑ EGTFTSDVSSYLEGQAAKEFIAWLVKGR	Orskov, Bersani, Johnsen, Hojrup, and Holst (1989))
GIP(1-42)	YA ↑ EGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ	(Sparre-Ulrich et al. (2017))
PYY(1-36)	YP ↑ IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY	Grandt et al. (1994))
NPY(1-36)	YP ↑ SKPDNPGEDAPAEDMARYYSALRHYINLITRQRY	Tatemoto (1982)
CXCL12α(1-67)	KP ↑ VSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	Dong et al. (2012)

↑ DPP4 proteolytic site

ligands in both *in vivo* biological and *in vitro* samples. Since the difference between intact and DPP4-cleaved ligands is only 2 amino acids, finding or designing a good antibody that only reacts with one of the two forms is problematic. Until last year, only GIP out of the five mentioned DPP4 substrates had an antibody that can differentiate between intact or full length GIP, and DPP4-cleaved GIP, this antibody is utilized in an ELISA assay to quantify GIP(1-42) (Troutt et al., 2011). In 2017, Bromage et al., managed to design a novel recombinant antibody that can be used in an ELISA assay to specifically quantify intact (non-DPP4 cleaved) CXCL12, with reportedly no cross-reactivity to DPP4-cleaved CXCL12 (Bromage, Taferner, Pillai, Yellon, & Davidson, 2017). Recently, our lab has generated a DPP4-cleaved CXCL12 specific polyclonal antibody that does not cross-react with the intact form and can detect physiological levels of DPP4-cleaved CXCL12 (Elmansi et al., 2018). Other commonly available antibodies that recognize DPP4 substrates are intended to detect the intact (non-DPP4 cleaved) form, but have not been characterized as to whether they also recognize proteolytic isoforms, or if they have been characterized, they see the intact and multiple N-terminal proteolytic variants (Antonsson, De Lys, Dechavanne, Chevalet, & Boschert, 2010; Baerts et al., 2015; Bromage et al., 2017; Richter et al., 2014; Wang et al., 2014). Quantification methods based on these antibodies misrepresent how much intact or cleaved ligand are in a biological sample and result in inaccurate or misleading results. A good example for misleading associations that resulted from not having the right quantification tools is the association between remote ischemic conditioning (RIC) and upregulated levels of CXCL12 (Fortunato et al., 2013; Jiang et al., 2013; Kamota et al., 2009). Using an inflatable cuff, RIC applied to either arms of healthy human volunteers, or to hind limps of Sprague-Dawley rats, and utilizing their new intact CXCL12-specific antibody; Bromage et al., demonstrated that although there is an upregulation in total CXCL12 levels as reported by previous literature, there is an unexpected significant decrease in intact CXCL12 levels after RIC, suggesting a dramatic increase in CXCL12 N-terminal cleavage following RIC and potentially flipping the interpretation of study results while revealing the presence of DPP4 isoforms that may themselves be influencing the results. Baerts et al, found similar results in ischemic heart disease (Baerts et al., 2015). These cautionary findings are applicable to all studies of DPP4 substrate ligands, but most especially where the metabolite products may have their own biological activity (Mulvihill & Drucker, 2014).

To avoid the daunting challenge of designing an antibody that is specific for either the intact or DPP4-cleaved ligand researchers turned to using different mass spectrometric techniques. Mass spectrometry (MS) characterizes and quantifies different peptides depending on their mass, making a two-amino acids difference easier to detect. The problem, however, is that in a complex biological sample, there is a vast pool of peptides that might share similar molecular weights. A second problem is the very low physiological concentration of some of the peptides in question are below the MS detection limits, resulting in the need to artificially add superphysiological concentrations of the ligands to biological samples to produce enough of the intact and cleaved variants to be detected using standard MS (Busso et al., 2005; De La Luz Sierra et al., 2004). These two challenges in MS analytical methods have confined it for a long time to qualitative analysis, which is identification and characterization of different cleaved variants of specific peptides under very limited conditions (Richter et al., 2014). To increase selectivity and obtain a lower physiological limit of detection, Chappell et al., utilized immunoaffinity enrichment MS with liquid chromatography sample purification to be able to quantify both GLP-1(7-36) and GLP-1(9-36) in human plasma (Chappell et al., 2014). Another pioneering paper is CXCL12 quantification by Wang et al., followed the same general scheme to accurately quantify intact and DPP4-cleaved CXCL12 in circulation of mice and rhesus monkeys (Wang et al., 2014). Wang et al., found that a DPP4 inhibitor while decreasing total levels of CXCL12, measured through other commercially-available quantification methods, actually significantly increased levels of the intact CXCL12; a major unexpected finding that demonstrates the importance of measuring intact and cleaved peptides in interpreting experimental data. This effect was speculated by the authors to represent a reduction in the overall amount of proteolytic isoforms while retaining or slightly increasing the amount of intact CXCL12. The inhibition of CXCL12 turnover results in negative homeostatic feedback regulation of the synthesis and release of CXCL12 by CXCR4^{+} cells that would normally increase CXCL12 synthesis when there is a reduction in intact CXCL12/CXCR4 signaling following proteolysis. The overall effect would be a decrease in total CXCL12 levels (Wang et al., 2014).

Even with having the right specific antibody, or the right immunoaffinity enrichment mass spectrometry protocol with a low enough limit of detection that works for biological samples, there is still the problem of very short half-life for some of these DPP4 substrates, and the importance of inhibition of DPP4 proteolysis during sample handling to allow accurate measurement of the substrates and their metabolic products. Utilizing labeled peptides, Kieffer et al., demonstrated that more than 50% of GLP-1 is metabolized in rats to DPP4-cleaved form within 2 minutes (Kieffer, McIntosh, & Pederson, 1995). Antonsson et al., reported 80% of CXCL12 was cleaved in plasma by DPP4 within 5 minutes. Interestingly, they also showed that the DPP4 cleaved isoform has a significantly longer half-life than full length CXCL12 and consequently may have a longer time for its own biological actions to be induced than the intact ligand (Antonsson et al., 2010). Baerts et al., analyzed immunoreactivity of CXCL12α in human plasma samples immediately after sample collection with, or without, DPP4 inhibitors in the collection tube. They showed a 25% decrease in the total amount of detectable CXCL12 when DPP4 was not inhibited at the time of collection. This would include not only intact CXCL12, but the DPP4-cleaved isoform and other further proteolytic isoforms. Importantly with further incubation (one hour) the total detectable CXCL12 dropped by 75% (Baerts et al., 2015). The results of this experiment and others show that *ex vivo* DPP4 proteolysis of CXCL12, and by extension other DPP4 substrates, can lead to misleading experimental data, both immediately at the time of collection or following the time needed for processing samples prior to analysis or storage (Antonsson et al., 2010; Baerts et al., 2015; Bromage et al., 2017; Hall, 2014; Mortier, Gouwy, Van Damme, & Proost, 2011; Richter et al., 2014; Wang et al., 2014). This raises an important and generally overlooked point; when it comes to DPP4 substrates quantification, sample collection and handling can greatly affect results and lead to data misinterpretation. We recommend that specific DPP4 inhibitors such as sitagliptin or linagliptin should be used not only for sample collection and preservation but also used as an important control treatment group when analyzing results. We also recommend the potential addition of broad spectrum proteolytic cocktails in combination with DPP4 inhibitors to block further degradation of extant DPP4-cleaved substrate metabolites in samples. This will help dissect out the impact of the metabolites and see the "specific" effect of intact or cleaved ligands in animals and cell culture studies. Without the use of DPP4 inhibitors in an experiment, researchers are looking at the effect of mixed populations of intact and cleaved ligands on their outcome measures. If DPP4 substrates are used as treatments in experiments where the DPP4 metabolites may have separate bioactivity, the generation of those isoforms and their impact on outcome measures should be considered.

All of these factors make the task of quantifying the cleaved or intact peptide challenging, and hence it is harder to establish an association between a certain biological function or trait, and appropriately assigning it to the intact ligand, the DPP4-cleaved ligand or a summing of effects from a combination of isoforms.

Another factor that interferes with both quantification of ligands, and interpretation of results; is binding of intact or cleaved forms to ECM molecules like glycosaminoglycans (GAGs). It was found that both GLP-1 and GLP-2 can be stored in aggregates with GAGs (Maji et al., 2009). Different GAGs have also been shown to bind CXCL12 and inhibit its cardioprotectitive effect (Peterson et al., 2004; Ziarek

A. Challenges in sample collection

Without DPP4 inhibitor in serum sample collection tubes, ligands are processed within minutes which affects the concentration reported from the experiment. A tube containing DPP4 inhibitor will show higher levels of unprocessed ligand compared to one containing the same sample but without DPP4 inhibitor

With DPP4 inhibitor   Without DPP4 inhibitor

## B. Challenges in quantification techniques

I %

m/z

Antibody-based methods: Difficulty in designing an antibody that can selectively detect one of the two isoforms (intact vs. cleaved), with only 2 amino acids difference between both isoforms
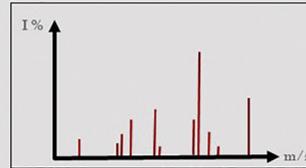
Mass-spectrometry based methods: Designing a method that quantify proteins in heterogeneous biological fluids without interference from other similar sized proteins, and at a very low physiological level can be challenging

C. Challenges in data interpretation

Ligand

GAG   Cell
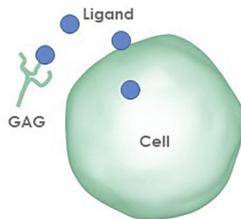
Difficulty in analyzing levels of soluble ligand compared to cell surface-bound or GAG-bound ligand. Also the challenges in specifically quantifying intact or cleaved ligand makes it more difficult to attribute a certain biological function to either isoforms

Fig. 1. Challenges in studying DPP4-cleaved substrates can be classified into challenges related to sample collection, quantification of cleaved substrate, or data interpretation

et al., 2013). This indicates that levels of other circulating factors like GAGs as well as GAGs in the extracellular microenvironment can affect the concentration and function of different cytokines and peptide hormones (Fig. 1). Importantly this may impact the amount of DPP4 substrate ligands that are available to be measured both due to GAG-ligand binding removing them from the soluble pool to be quantified, as well as masking them from antibody-based assays by blocking antigenic sites targeted by the antibodies. Some investigators are now also trying to liberate GAG-bound DPP4-substrate ligands in samples prior to quantification to be able to assess the different ligand pools (Irhimeh, Fitton, & Lowenthal, 2007; Sweeney & Papayannopoulou, 2001).

All of the previous factors demonstrate how complicated and challenging the study of DPP4 substrates can be. However, with the right experimental tools, study design, and data interpretation based on critical thinking, researchers have been able to start to show that many DPP4-cleaved peptides possess separate bioactivity from their parent peptides. In the next section, we will present their data and try to connect the dots between different results to form a better understanding of how DPP4 changes its substrates' bioactivity.

3. GLP-1

GLP-1(7-36) is a peptide hormone released from the L cells of the small intestine in response to food ingestion. GLP-1 hormone binds to the GLP-1 receptor (GLP-1R) and results in stimulating insulin secretion as well as inhibiting glucagon release. As a result, the blood glucose level usually decreases following food ingestion (Nauck, 2013). GLP-1

possesses additional, non-gastrointestinal related effects. These effects include protection against post-myocardial infarction remodeling and inhibition of adipose stem cells (ASC) differentiation. A higher dose of GLP-1 leads to significant inhibition of both ASC cell count and cell growth (Chen, Angeli, Shen, & Shannon, 2014; Goodwill et al., 2014; Sauve et al., 2010; Sokos, Nikolaidis, Mankad, Elahi, & Shannon, 2006).

Full length GLP-1 has a limited half-life prior to DPP4 cleavage. GLP-1 injected subcutaneously in beagle dogs has a short half-life of less than 60 minutes prior to being cleaved by DPP4 into GLP-1(9-36). In rats the half-life prior to DPP4 cleavage has been reported to be as short as 2 minutes (Kieffer et al., 1995). For a long time, GLP-1(9-36) was considered to be inactive (Knudsen & Pridal, 1996). However, a recent study by Chen et al., showed that it possesses some of the functional activity of intact GLP-1 (Chen et al., 2014).

3.1. GLP-1 effect on postprandial glycemia:

Studies have shown that exogenous administration of either intact or DPP4-cleaved GLP-1 to human subjects leads to reduction of both fasting and postprandial glucose levels. However, the effect of intact GLP-1 was different from the cleaved. It has been found that the cleaved form had minimal effect on reducing blood glucose levels, in addition that effect was only seen with the use of supraphysiological levels of cleaved GLP-1. In contrast, intact GLP-1 reduced blood glucose level much more efficiently and that effect was attributed to delaying gastric emptying and lowering glucagon levels. Cleaved GLP-1 had no significant effect on either gastric emptying or glucagon levels (Meier et al., 2006; Vahl, Paty, Fuller, Prigeon, & D'Alessio, 2003).

These findings were further confirmed by another study in pigs conducted by Deacon et al. The aim of the study was to assess the effect of cleaved GLP-1 on blood glucose levels. The study found that both intact and cleaved GLP-1 reduced blood glucose. They also suggested that cleaved GLP-1 works through enhancing the glucose disposal rate with no effect on insulin secretion (Deacon et al., 2002). Another study by Cantini et al., was conducted to evaluate the effect of intact and cleaved GLP-1 on glucose uptake. To do that, they treated ASCs for 1 hour with 10 nM insulin in the presence or absence of 10 nM of either intact or cleaved GLP-1. They found that both intact and cleaved GLP-1 significantly inhibited glucose uptake to a similar extent (Cantini et al., 2017).

Altogether, it has been strongly suggested that the cleaved GLP-1 form shares some effects with intact GLP-1, but has significant differences in how it achieves those effects. Interestingly, it has been reported that the cleaved form might be signaling and acting through receptors other than GLP-1R, which would explain the different mechanisms of action (Cantini, Mannucci, & Luconi, 2016).

3.2. GLP-1 effect on adipogenesis

Intact GLP-1 is known to inhibit ASC adipogenesis, and to downregulate the expression of adipocyte-specific markers like adipocyte protein 2, lipoprotein lipase and peroxisome proliferator-activated receptor gamma (El Bekay et al., 2016; Lee et al., 2015).

Cantini et al., evaluated the effect of intact and cleaved GLP-1 on ASC adipogenesis in vitro using Oil Red O and AdipoRed stains. Their results showed that treating ASCs with either intact or cleaved GLP-1 reduced the number of differentiated adipocytes as well as cytoplasmic lipid droplets. Also, both intact and cleaved GLP-1 reduced mRNA expression of FABP4, an adipose differentiation marker. They found that exendin (9-39), a competitive antagonist of GLP-1R, was able to reverse only the intact GLP-1 effect, but not that of cleaved GLP-1. This suggests that both intact and cleaved GLP-1 are working through different receptors (Cantini et al., 2017) (Table 2).

3.3. GLP-1 effect on apoptosis and cell proliferation

Both intact and cleaved GLP-1 significantly inhibited cell growth and decreased cell count. Exendin (9-39) itself did not affect cell numbers. However, it was able to reverse the decrease in cell numbers that was caused by intact GLP-1, but not cleaved GLP-1. Cytofluorimetric assays of annexin-V exposure on cell surface showed that both intact and cleaved GLP-1 significantly induced cellular apoptosis and caused a significant increase in apoptotic ASCs. In addition, both forms inhibited cell proliferation as shown by results of MTT assays. Similarly, exendin (9-39) was able to reverse the apoptotic effect of the intact GLP-1 with no significant action on cleaved GLP-1's effect (Cantini et al., 2017).

3.4. GLP-1 effect on the cardiovascular system

The effects of intact and cleaved GLP-1 on the cardiovascular system have been assessed in different models. Ex vivo, rat thoracic aortas were excised and cut into 3 mm rings to assess cumulative relaxation responses for both intact and cleaved GLP-1. Both intact and cleaved GLP-1 significantly relaxed the rat aorta vasculature in a dose-dependent manner (Green et al., 2008).

In vivo, intact GLP-1 is known to protect myocardial tissue from ischemic events, an effect that GLP-1R antagonist exendin (9-39) was not able to reverse. The myocardial protective effect of GLP-1 even persisted in GLP-1R knockout mice (Ban et al., 2008; Sonne, Engstrøm, & Treiman, 2008). Cleaved GLP-1, on the other hand, was shown to be specifically protective against the development of diastolic dysfunction after myocardial infarction. It also reduced myocardial inflammation by affecting infiltrating macrophages. In addition, cleaved GLP-1 increased mitral valve E/A ratios, and decreased E wave deceleration rate in echocardiography in mice with myocardial infarction. These protective effects of cleaved GLP-1 were not reversed by exendin (9-39) (Robinson et al., 2016). Accordingly, it is now starting to be thought that this protective effect against cardiac ischemia can be attributed exclusively to cleaved GLP-1, and that previously it was mistakenly attributed to intact GLP-1. An experiment that proved this point was conducted by Ban et al., where sitagliptin, a DPP4 inhibitor, was used to prevent the truncation of the intact GLP-1 resulting in diminished intact GLP-1 protective effects against ischemia (Ban et al., 2008; Sonne et al., 2008).

3.5. GLP-1R Receptor binding

Based on findings of the studies mentioned above, cleaved GLP-1 possesses physiological actions that are not inhibited by blocking the GLP-1R or knocking down its expression. This suggests that cleaved GLP-1's actions may be through another pathway independent of the classical GLP-1R pathway (Fig. 2). This provides a strong rationale to perform receptor binding assays of both intact and cleaved GLP-1 to GLP-1R. These studies will be essential in the interpretation of various experimental results.

To determine affinity and binding density of both intact and cleaved GLP-1 to GLP-1R, Kuc et al., performed radioligand binding assays on coronal sections of mouse brain. These sections were selected since the tissues have high expression levels of GLP-1R as indicated by the localized in situ hybridization expression of its mRNA. Their results showed that both binding affinity (K_D) and binding density (B_{MAX}) of cleaved GLP-1(9-36) to GLP-1R in mouse brain coronal sections were significantly lower than intact GLP-1(3-36). Moreover, scintillation proximity assays (SPA) showed differential binding of intact and cleaved GLP-1 to GLP-1R. Based on SPA, the affinity of cleaved GLP-1 to GLP-1R receptor (Ki) was 70,000-fold less than intact GLP-1. Finally, cAMP assays were performed in CHO-K1 cell line expressing wild type GLP-1R receptors to evaluate receptor activation. In line with minimal association and binding of cleaved GLP-1 to the GLP-1R receptor, the cAMP assay results showed minimal signaling activation of GLP-1R by cleaved GLP-1. The maximum intracellular signaling of cleaved GLP-1 (E_{MAX}) was 10% of the intact form, and an agonist response curve showed that almost 10^5 fold increase in cleaved GLP-1(9-36) concentration was required to reach this E_{MAX} as shown by EC_{50} values (Kuc et al., 2014) (Table 3). This supports the idea that most - if not all - of cleaved GLP-1's biological effects are mediated through a different receptor than GLP-1R.

Table 2
Biological functions of intact and cleaved GLP-1 alone and with exendin(9-39) (GLP-1R blocker)

| Effect	Intact GLP-1(7-36)		Cleaved GLP-1(9-36)		Ref.
	Alone	With exendin(9-39)	Alone	With exendin(9-39)	
Cell count	Decrease	No decrease	Decrease	Decrease	Cantini et al. (2017)
Cell proliferation	Decrease	ND	Decrease	ND	Cantini et al. (2017)
Apoptosis	Increase	ND	Increase	ND	Cantini et al. (2017)
Adipogenesis	Decrease	No decrease	Decrease	Decrease	Cantini et al. (2017), El Bekay et al. (2016)
Glucose uptake	Decrease	ND	Decrease	ND	Cantini et al. (2017)
Myocardial protective effects	Increase	Increase	Increase	Increase	Robinson et al. (2016), Sonne et al. (2008)

ND: not determined
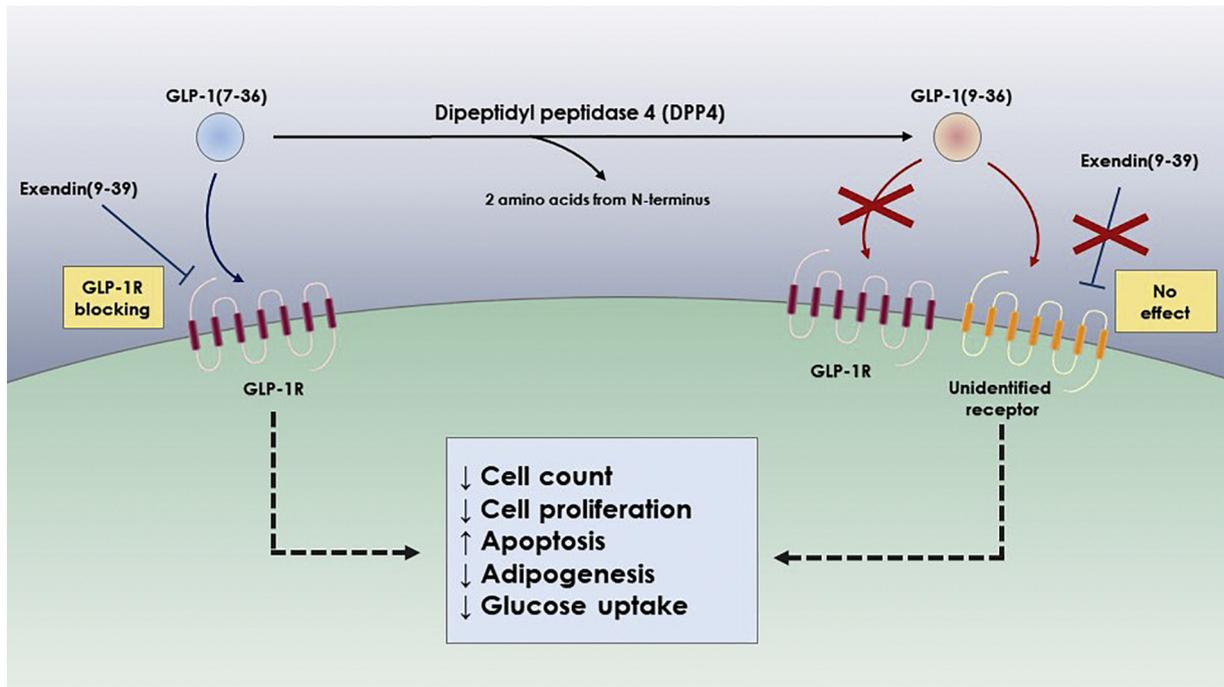
Fig. 2. Effect of DPP4 proteolysis on receptor activation and biological functions of GLP-1

3.6. GLP-1 summary

While DPP4-cleaved GLP-1(9-36) displays some of the intact ligand's biological functions, we believe that the cleaved form is acting through a different mechanism than intact GLP-1(7-36) since the effects are not blocked by GLP-1R inhibitors like exendin or by knock-down of the receptor. Of all the important biological functions that intact GLP-1 influences, such as decreasing ASCs count and proliferation, increasing apoptosis, and downregulating glucose uptake, we think there is evidence that GLP-1's myocardial protective function against ischemic events and diastolic dysfunction is in fact due to DPP4-cleaved GLP-1 (9-36) mediated through a receptor other than GLP-1R.

4. GIP

GIP is a 42 amino acid long peptide hormone. It is synthesized by enteroendocrine K-cells of the stomach and duodenum. Similar to GLP-1, GIP is responsible for regulation of postprandial glycemia (Elahi, 1994). Structurally, the C-terminal domain is dispensable for GIP effects. As a matter of fact, the functional activity of GIP wasn't reduced after removing three to four amino acids from the C-terminal. Moreover, the GIP(1-31) fragment, missing 11 amino acids from the C-terminal, was still able to retain cAMP stimulatory effect equal to that of intact GIP (Fujita et al., 2010; Fujita, Asadi, Yang, Kwok, & Kieffer, 2010). On the contrary, the N-terminal is critical for the activity of GIP-1. It has been found that while recombinant GIP missing 4 amino acids from the N-terminal domain was able to bind to the receptor with

a similar affinity to the intact form, its insulintropic activity was significantly reduced (Hinke et al., 2001). As a DPP4 substrate, DPP4 truncates GIP peptide at its N-terminal by removing the two terminal amino acids. Lacking the N-terminal essential for its signaling activity, cleaved GIP was believed to be biologically inactive (Deacon, 2004; Kieffer et al., 1995). However, its ability to still bind the receptor suggests it may act as a competitive inhibitor.

Normally GIP is cleaved on both the N and C terminals though depending on the cleavage site the activity of the resulting fragment significantly varies. We are going to be discussing how DPP4 N-terminal cleavage affects both intact GIP(1-42) and C-terminal cleaved GIP(1-30), converting them into GIP(3-42) and GIP(3-30), respectively.

4.1. GIP effect on glucose regulation

In ob/ob mice, intact GIP(1-42) results in decreased plasma glucose after glucose administration. In contrast GIP(3-42) leads to an initial increase in glucose reaching its peak after 15 minutes, before it starts to decrease. Administration of other N-terminal cleaved GIP isoforms to ob/ob mice, like GIP(8-42), also showed a significant inhibition to intact GIP glucose lowering effect (Kerr, Flatt, Flatt, & Gault, 2011).

4.2. GIP effect on Insulin secretion

In pancreatic BRIN-BD11 cells, intact GIP stimulated insulin secretion in a dose-dependent manner. GIP (3-42) on the other hand significantly decreased insulin secretion along with other N-terminal truncated variants including GIP(4-42) and GIP(8-42). When intact GIP was co-

Table 3
Receptor binding assays of intact and cleaved GLP-1 to GLP-1R

	Intact GLP-1(7-36)	Cleaved GLP-1(9-36)	Ref.
Saturation binding assay K_D	1.29 ± 0.26 AU	0.214 ± 0.08 AU	Kuc et al. (2014)
Saturation binding assay B_{MAX}	57.0 ± 14.5 AU	2.69 ± 0.74 AU	Kuc et al. (2014)
SPA binding assay K_i	0.102 nM	156 nM	Kuc et al. (2014)
cAMP assay EC_{50}	0.072 nM	7270 nM	Kuc et al. (2014)
cAMP assay E_{MAX}	104%	8.93%	Kuc et al. (2014)

Table 4
Effect of C-terminal and N-terminal cleavage on receptor binding and biological functions of GIP

Effect	GIP(1-42)	GIP(3-42)	GIP(1-30)	GIP(3-30)	Ref.
GIP Receptor binding	High	Low	High	High	Hansen et al. (2016)
Receptor activity	Agonist	Weak antagonist	Agonist	Strong antagonist	Hansen et al. (2016)
cAMP production	Increase	Decrease	Increase	Decrease	Kerr et al. (2011)
Insulin secretion	Increase	Decrease	Increase	Decrease	Kerr et al. (2011), Sparre-Ulrich et al. (2017)

administered with GIP(8-42), another N-terminal truncated variant, GIP (8-42) significantly decreased intact GIP-induced insulin release at 15 and 30 minutes after administration. Also, when cells were incubated with either GIP(3-42) or GIP(8-42), together with intact GIP, insulin secretion stimulated by intact GIP was significantly inhibited (Kerr et al., 2011). This could mean either that N-terminal truncated GIP variants possess an antagonistic effect to intact GIP through the same receptor by competitive inhibition, or via another unidentified receptor.

In vivo, treatment with different variants of N-terminal truncated GIP did not significantly alter plasma insulin response after glucose injection in ob/ob mice, while intact GIP significantly increased plasma insulin level (Kerr et al., 2011). In isolated perfused rat pancreas, GIP (3-30) competitively inhibited GIP(1-42) insulin, glucagon, and somatostatin secretion (Sparre-Ulrich et al., 2017) (Table 4). The reason for different results in isolated perfused rat pancreas versus the whole mice is not explained, but it could be due to the fact that *in vivo* serum insulin level is more tightly controlled than secreted insulin level in an isolated pancreas.

4.3. GIP effect on cAMP response

In terms of receptor activation, Hansen et al showed that cAMP activation in response to GIP receptor activation was almost the same for both GIP (1-42) and GIP(1-30) in COS-7 cells, which are fibroblast-like cell lines derived from monkey kidney tissue. This suggests that the C-terminal of GIP is not essential for GIP receptor activation and down-stream responses. However, Kerr et al., found that DPP4 N-terminal truncated GIP (3-42) had a significantly lower cAMP expression, when compared to intact GIP in BRIN-BD11 cells (Hansen et al., 2016; Kerr et al., 2011). This shows again that the N-terminal is necessary for receptor activation and subsequent cAMP activation, and that

cleavage of N-terminal leads to loss of signaling events downstream to GIP receptor activation. However, the mechanism of ligand inactivation versus antagonism is important to distinguish.

4.4. GIP receptor binding

Hansen et al., demonstrated that the N-terminal was critical for high-affinity binding to GIP receptor (GIP-R). The C-terminal on the other hand was a modulator for agonistic versus antagonistic actions (Fig. 3). It was found that GIP(3-30) acted as a GIP-R antagonist in both human and rat cells. GIP(3-30) competitively inhibited GIP receptor activation by the intact form GIP(1-42). A very interesting finding was that although GIP(3-42) did not possess any agonistic activity, it was a significantly less potent antagonist than GIP(3-30) both in human and rat systems, suggesting that the C-terminal can be acting as a negative regulator of the antagonist action. Competitive binding assays for GIP(1-30) and GIP(3-30) were performed to compare affinity of both ligands to the GIP-R. It was found that DPP4-cleaved GIP(3-30) had a lower $\log(IC_{50})$ and higher K_i, suggesting it had a higher affinity to bind to the GIP-R than GIP(1-30) (Hansen et al., 2016) (Table 5).

4.5. GIP summary

GIP is one of the most intriguing DPP4 substrates. While intact GIP (1-42) is a critical incretin hormone that increases insulin and glucagon secretion, N- and C-terminals cleaved GIP(3-30) results in strong antagonism to GIP-R mediated functions. It is believed that the N-terminus, cleaved by DPP4, controls whether GIP variants are agonistic or antagonistic to GIP-R signaling, while the C-terminus controls the strength of the antagonistic effect of the N-terminal DPP4 cleaved GIP on the GIP-R.
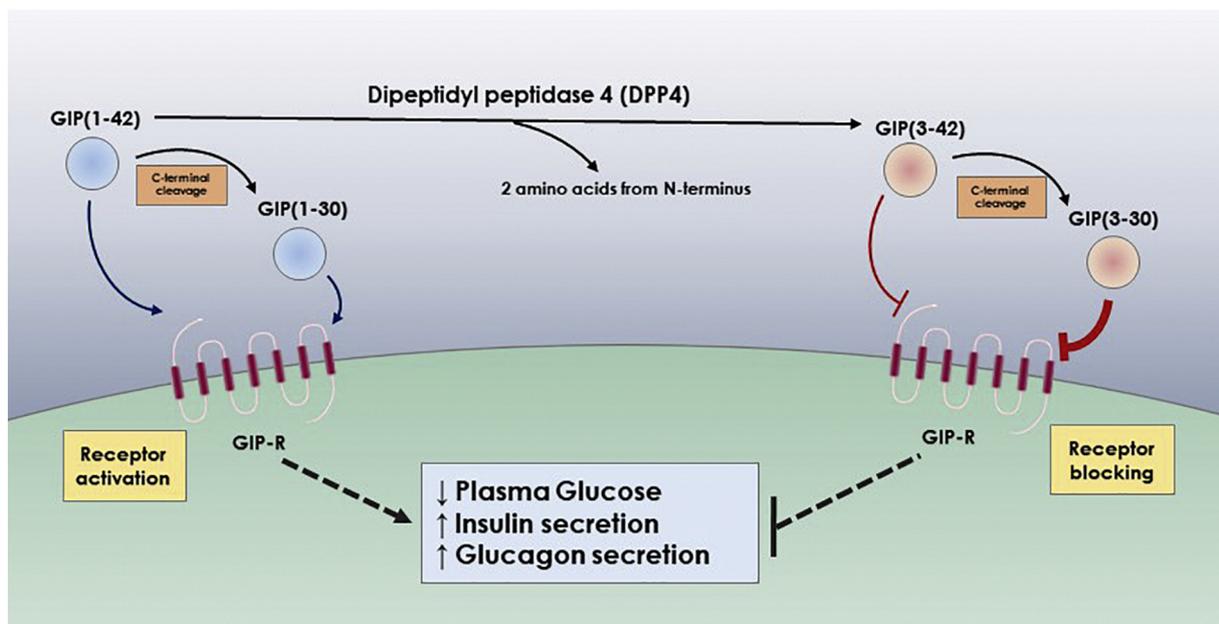

Fig. 3. Effect of DPP4 proteolysis on receptor activation and biological functions of GIP

Table 5
Receptor binding assays of GIP(1-30) and GIP(3-30) to GIP receptor

	GIP(1-30)	GIP(3-30)	Ref.
Competitive binding assay logIC$_{50}$	-9.05 ± 0.02 AU	-8.63 ± 0.04 AU	Hansen et al. (2016))
Competitive binding assay K$_i$	0.89 nM	2.3 nM	Hansen et al. (2016)

5. PYY

PYY is a 36 amino acid member of the PP-fold family of peptide hormones. It is released from the distal intestine along with GLP-1 during digestion (Adrian et al., 1985). This family of peptides act preferentially through binding specific receptors known as Y1, Y2, Y4, Y5, and Y6. Similar to the intestine, PYY is also expressed in the brain, where it binds at least three PYY receptors (Y1, Y2, and Y5) with different affinities (Blomqvist & Herzog, 1997). PYY exerts a wide variety of physiological actions, the most distinct being its ability to decrease appetite and inhibit food intake. In addition, it also inhibits gastrointestinal motility, gastric and pancreatic secretions, decreases glomerular filtration rate and promotes vasoconstriction (Ferrier, 2000; Playford et al., 1992; Playford et al., 1995; Yang, 2002).

5.1. Y receptors subtypes

The different actions of PYY are achieved through binding to different receptors. Y1 and Y5 activation is responsible for triggering hunger and increasing appetite. Interestingly, Y2 receptor activation decreases appetite and consequently decreases body weight in mice (Gerald et al., 1996). Another main functional contrast between Y1 and Y2, is that Y1 specific agonists decrease anxiety, while Y2 specific agonists are anxiogenic. Receptors also vary in their distribution pattern within different tissues. Y1 receptor is expressed in adipocytes, colon, vascular smooth muscles, and the cerebral cortex (Castan et al., 1993; Dumont, Fournier, St-Pierre, Schwartz, & Quirion, 1990; Grundemar et al., 1992; Mannon, Mervin, & Sheriff-Carter, 1994). It is involved in the regulation of decreased anxiety and depression (Caberlotto et al., 1999; Wahlestedt, Pich, Koob, Yee, & Heilig, 1993). The Y2 receptor is expressed in nerve fibers, the hippocampus, and intestine (Gehlert, Gackenheimer, & Schober, 1992; Rettenbacher & Reubi, 2001; Stjernquist & Owman, 1990), and it is associated with memory retention and angiogenesis (Flood & Morley, 1989; Zukowska-Grojec et al., 1998). The Y5 receptor is expressed in the hypothalamus, intestine, ovary, testis, pancreas, and skeletal muscle (Gerald et al., 1996; Herzog et al., 1997). It is involved with stimulation of appetite, and regulation of brain seizures and circadian rhythm (Gerald et al., 1996; Gribkoff, Pieschl, Wisialowski, van den Pol, & Yocca, 1998; Guo, Castro, Palmiter, & Baraban, 2002; Matsumoto, Basil, Jetton, Lehman, & Bittman, 1996).

DPP4 cleaves PYY (1-36) and yields PYY (3-36). PYY (3-36) has been proven to possess a distinct biological activity. Grandt et al., showed that DPP4-cleaved PYY(3-36) is a selective Y2 receptor agonist, as compared to intact PYY(1-36) that binds to both Y1 and Y2 receptors (Grandt et al., 1994). This shows a very interesting effect for DPP4 on PYY, where it shifts it from a limited-selective multi-receptor agonist that binds to three different Y receptors, to a highly selective agonist that binds only to one of these receptors. Based on that, there is a great chance

that cleaved PYY (3-36) would be able to show a more prominent pattern of Y2 receptor activation compared to PYY (1-36). Therefore, DPP4 may be implicated in physiological control of appetite, by decreasing hunger-triggering Y1 and Y5 receptor signals, and upregulating anxiogenic Y2 receptor signal. Further studies, however, need to be conducted to support this hypothesis, and to test if pharmacological DPP4 inhibitors lead to increased food intake by downregulating PYY(3-36) protein levels and how this might interact with DPP4 mediated insulin and glucose regulation.

5.2. PYY effect on food intake

According to a study conducted by Unniappan et al., they showed that in normal rats, both PYY (1-36) and PYY (3-36) significantly reduced 24-h food intake. In contrast, in DPP4 deficient rats, PYY(3-36) significantly reduced 24-h food intake, while PYY(1-36) had no notable effect (Unniappan et al., 2006) (Table 6). This result suggests another instance where the effect of the DPP4-cleaved substrate may be mistakenly attributed to an intact form of the parent ligand. Without proper inhibition of the degrading enzyme, effects of both intact and cleaved form may be confused (Fig. 4).

After fasting, DPP4 deficient rats also showed a higher 24-h food intake and weight gain. They also showed higher cumulative food intake during light phase and dark phase (Unniappan et al., 2006). It has been also shown that treatment with PYY(3-36) through various routes of administration in different rat, mouse, and rhesus monkey models, in addition to obese humans, lead to reduction of food intake (Batterham et al., 2003; Chelikani, Haver, & Reidelberger, 2005; Moran et al., 2005; Pittner et al., 2004; Tschop et al., 2004). Taken together, these results support the idea that inhibition of food intake may in part be caused by the DPP4-cleaved form of PYY (3-36).

5.3. PYY effect on GIT motility

Moriya et al., showed that PYY (3-36) - in addition to other Y2 receptor agonists – significantly inhibited castor oil-induced diarrhea in a dose-dependent manner in mice. PYY (3-36) also significantly blocked 16,16-dimethyl Prostaglandin E2-induced intestinal fluid accumulation. It also significantly and dose-dependently reduced wet fecal weight and bead expulsion time by slowing colonic transit (Moriya et al., 2010). This is an effect that was not reported for PYY(1-36). This could be because PYY(3-36) is a pure Y2 receptor agonist, while the effects of PYY (1-36) is the result of Y1, Y2, and Y5 activation, which dilutes the overall effect producing a less distinct phenotype.

5.4. PYY effect on locomotor and exploratory behavior

PYY (3-36) effects are not only restricted to food intake and GIT motility. Stadlbauer et al., showed that mice treated with PYY (3-36) show

Table 6
Receptor selectivity and effect on food intake of intact and cleaved PYY

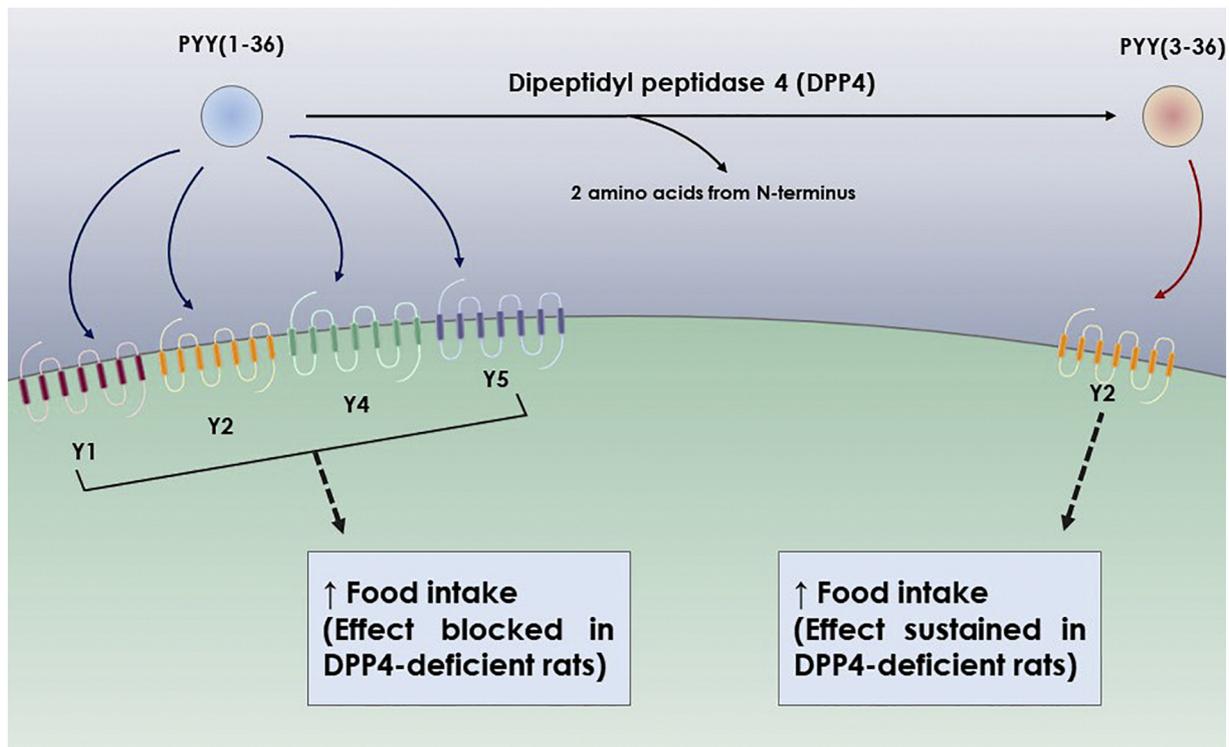|	Intact PYY(1-36)	Cleaved PYY(3-36)	Ref.
Receptor selectivity	Y1, Y2, Y4, and Y5 agonist	Y2 agonist	Grandt, Schimiczek, Beglinger, et al. (1994)
Food intake	Decrease (no effect on DPP4-deficient mice)	Decrease (acts on DPP4-deficient mice)	Batterham et al. (2003), Chelikani et al. (2005), Moran et al. (2005), Pittner et al. (2004), Tschop et al. (2004)

Fig. 4. Effect of DPP4 proteolysis on receptor activation and biological functions of PYY

increased novel object exploration compared to vehicle treated mice. Also, mice treated with PYY (3-36) showed a higher locomotor habituation response to novel environments than mice treated with vehicle alone. PYY (3-36) potentiated the increase in locomotor response to amphetamine, as measured by the distance moved in an open field. Apomorphine treatment induces stereotyped behavior in mice, which is evidenced by increased leaning and climbing. PYY(3-36) also increased this stereotyped apomorphine behavior (Stadlbauer, Weber, Langhans, & Meyer, 2013). These effects are also believed to be a result of Y2 receptor activation in the brain.

5.5. PYY summary

Intact PYY(1-36) activates 4 different receptors (Y1, Y2, Y4, and Y5), and as expected, the biological effects from activating these 4 different receptors is complicated and hard to accurately measure compared to PYY(3-36) which appears to work as a Y2-specific receptor agonist. The only well documented biological function where PYY(1-36) is compared to PYY(3-36) is the effect on food intake. Both intact and cleaved variants decrease 24-h food intake in rats, and only PYY(3-36) decreases 24-h food intake in DPP4-deficient rats. This suggests that the decrease in food intake following intact PYY(1-36) delivery is actually due to the DPP4 cleavage yielding PYY(3-36) after treatment in rats. Cleaved PYY also possesses other important biological activities on GIT mobility, and CNS mediated locomotor and exploratory behavior in mice. A better understanding of these functions as well as other possible effects of cleaved and intact PYY and interaction with other DPP4 cleaved substrate signaling pathways is necessary to assess benefits and drawbacks of using clinical DPP4 inhibitors for treatment of diabetic patients or in other populations.

6. NPY

NPY is another 36 amino acid peptide hormone that is a Y receptor family agonist. Similar to PYY, it is expressed in the hypothalamus and is truncated by DPP4 to also produce a selective Y2 receptor agonist.

NPY (1-36) increases short term 2-h food intake. In addition, NPY inhibits osteoblast activity centrally by binding to the Y2 receptor and peripherally by binding to Y1 receptor. NPY also affects adiposity both centrally in the hypothalamus and peripherally. Centrally, NPY increases fat accretion through Y1 and Y5 receptors leading to increased bone marrow adiposity, and decreases bone marrow fat accretion through the Y2 receptor. Bone marrow and peripheral adiposity are differentially regulated, therefore it is interesting that in peripheral tissues, NPY leads to increased fat accretion through activation of both Y1 and Y2 receptors. NPY also plays a role in increasing adhesion and chemotaxis of macrophages. It also contributes in synaptic transmission and modulates neuroplasticity. NPY affects a complex interdigitating network of biological functions through four different receptors, and a better understanding of how DPP4-cleaved PYY is contributing to this complex network is essential to grasp the extents to which DPP4 inhibitors are affecting neuropeptide biology.

6.1. NPY receptor binding

In 1996, Grandt et al., found that both NPY(1-36) and NPY(3-36) compete in binding to CHP 234 human neuroblastoma cells; a type of cell that expresses the Y2, but not the Y1 receptor, on their surface. However, SK-N-MC human neuroblastoma cells, which in contrast express Y1 receptor, but not the Y2 receptor, only bind NPY(1-36). These results suggest that similar to PYY; the intact form is a non-selective agonist for both Y1 and Y2 receptors, while the cleaved form is a selective Y2 agonist (Grandt et al., 1996) (Fig. 5).

6.2. NPY effect on cell migration

It has been found by Ghersi et al., that both NPY(1-36) and NPY(3-36) cause human umbilical vein endothelial cells (HUVECs) to migrate in a wound healing model. However, when DPP4 activity was inhibited using a DPP4 neutralizing mAb the anti-migratory effect of NPY(1-36) was significantly decreased. This suggests that increasing cell migration in wound healing model may be due to DDP4
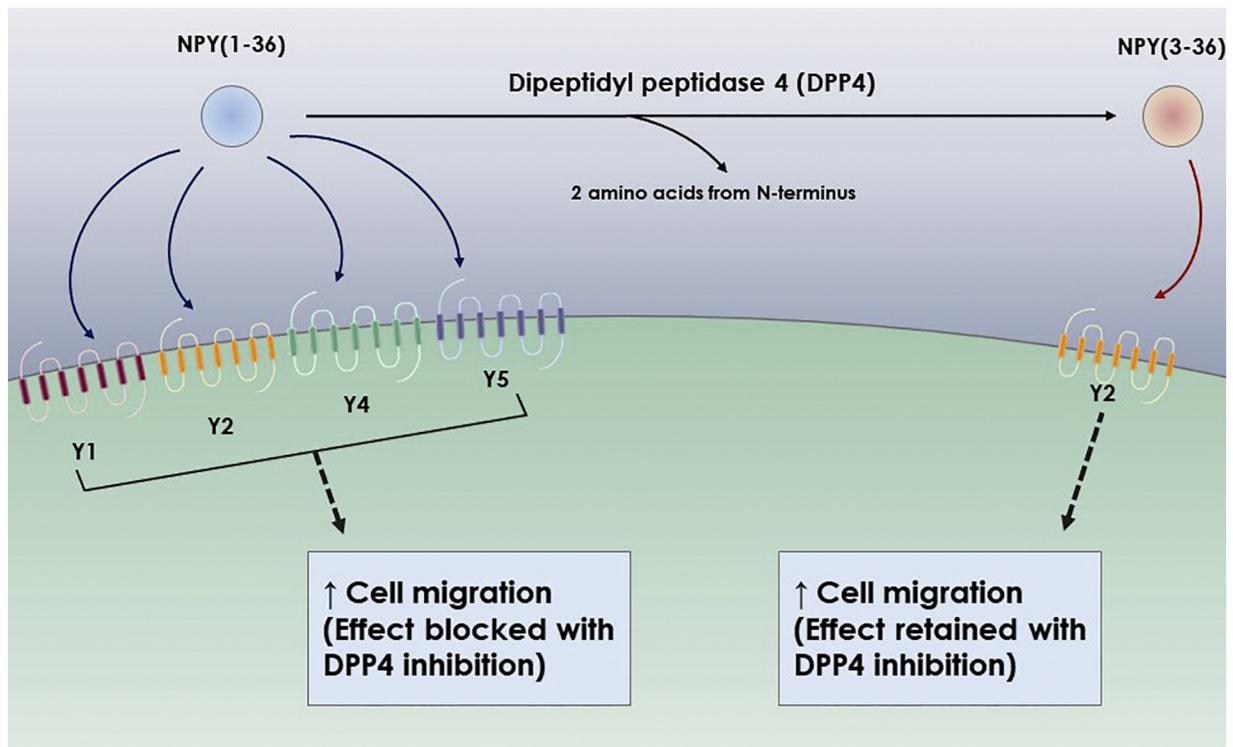
Fig. 5. Effect of DPP4 proteolysis on receptor activation and biological functions of NPY

cleaved NPY(3-36) rather than the intact form (Ghersi, Chen, Lee, & Zukowska, 2001) (Table 7).

Moreover, Singh et al reported that in the hematopoietic microenvironment, DPP4-cleaved NPY(3-36) reduced VE-cadherin and CD31 expression in endothelial cell junctions, and mobilized hematopoietic stem and progenitor cells (HSPCs) through Y2 and Y5 receptor activation. HSPCs trafficking was impaired in mice lacking either DPP4 or NPY, and was restored upon treatment of mice with DPP4-cleaved NPY(3-36) (Singh et al., 2017).

6.3. NPY(3-36) effect on food intake

The effect of NPY(3-36) on food intake has not been as thoroughly studied as PYY(3-36). One publication comparing NPY(1-36) to NPY(3-36) in rats in the context of food intake showed that NPY(3-36) leads to a greater increase in 2-hour food intake compared with NPY(1-36). The increase in food intake was found to be dose-dependent and it plateaued as the dose increased beyond 2.5 μg/rat (Flynn et al., 1998) (Table 7). This is an unexpected result, and is in contrast to the effects of PYY(3-36) on food intake. An explanation for why NPY(3-36), a Y2 selective agonist, leads to increased food intake is unclear, and further work needs to be done to assess this effect of NPY(3-36) on a longer timeline. This suggests DPP4-cleaved PYY and NPY can differentially act on the Y2 receptor.

6.4. NPY summary

NPY is not as thoroughly studied as GLP-1 or GIP, but it has been shown that intact NPY(1-36) can activate 4 different Y receptors (1, 2, 4, and 5) while DPP4-cleaved NPY(3-36) only activates the Y2 receptor. Both NPY(1-36) and (3-36) induce HUEVCs migration, but with DPP4 inhibition, only NPY(3-36) retains this effect, which suggests that NPY(1-36) can only induce cell migration after being cleaved by DPP4. Also, the effect of NPY on food intake is limited, however, in 2-h food intake studies DPP4-cleaved NPY(3-36) shows a greater inhibition of intake in rats compared to intact NPY(1-36).

7. CXCL12, stromal cell-derived factor 1 (SDF-1)

SDF-1/CXCL12 is a highly conserved pleiotropic chemokine/cytokine that is considered one of the earliest cytokines evolutionarily (Shirozu et al., 1995). It is produced by numerous cell types and tissues constitutively and its expression is induced by most tissues in response to injury (Herberg et al., 2013). Common sources are tissue stromal/support cells such as mesenchymal derived cells involved in stem cell niche organization, cardiomyocytes, neural stem cells (NSCs), osteogenic cells, and endothelial cells (Carbone et al., 2017b; Herberg, Fulzele, et al., 2013; Mortensen & Hill, 2015). For example, we and others have shown that in the brain astrocytes are the primary constitutive source of CXCL12. Astrocytes increase CXCL12 secretion during development regulating

Table 7
Receptor selectivity and effect on cell migration and food intake of intact and cleaved NPY

	Intact NPY(1-36)	Cleaved NPY(3-36)	Ref.
Receptor selectivity	Y1, Y2, Y4, and Y5 agonist	Y2 agonist	Grandt et al. (1996)
Cell migration	+ (effect blocked with DPP4 inhibition)	+(effect retained with DPP4 inhibition)	Ghersi et al. (2001)
2 Hour food intake	+	++	Flynn et al. (1998)

neuronal cell and process migration, as well as in maintenance of the organization of the adult neurogenic zone, and critically in tissue repair following injury (Hill et al., 2004; Miller et al., 2005; Shin et al., 2014). CXCL12 is also highly secreted by lung and bone marrow stromal cells, as well as stromal and endothelial cells in other tissues being involved in a large spectrum of biological and developmental processes (Bleul, 1996; Nagasawa et al., 1996). CXCL12 is involved with cardioprotection, bone repair, neuronal regeneration, tumor cell mobilization, migration and homing/metastasis (Bromage et al., 2014; Broxmeyer et al., 2005; Cheng, Wang, et al., 2017; Li, Basu, Han, Kim, & Broxmeyer, 2007; Yang et al., 2018). On the cellular level, CXCL12 affects migration, survival, and proliferation (Herberg et al., 2013; Leite Pereira et al., 2018; Reid et al., 2018). In humans CXCL12 has six alternative splice variants (α to φ) differing only in the exon related to the C-terminus. Out of these six, the most common splice variants are CXCL12α (1-68) and CXCL12β (1-72) (Yu et al., 2006). All CXCL12 variants, including CXCL12α and CXCL12β, are cleaved by DPP4 removing their N-terminus KP dipeptide amino acids (Kato et al., 1998; Lambeir et al., 2001). Importantly, the ability to initiate the DPP4 attack is regulated by the C-terminus. CXCL12α is rapidly cleaved when its C-terminal lysine is removed by Carboxypeptidase M or N, in contrast the half-life of the N-terminal intact CXCL12β is longer due to the protection of lysine 68 by the four additional C- terminal amino acids blocking the binding of the carboxypeptidases (Herberg, Fulzele, et al., 2013; Herberg, Shi, et al., 2013). Other than difference in half-life, there seems to be no significant difference in receptor selectivity or functions between CXCL12α and CXCL12β (De La Luz Sierra et al., 2004; Herberg, Fulzele, et al., 2013; Herberg, Shi, et al., 2013; McQuibban et al., 2001; Proost et al., 1998; Richter et al., 2014; Vergote et al., 2006). Interestingly for CXCL12, not only does DPP4 cleave two amino acids from its polypeptide structure, but it sets CXCL12 up for further proteolysis by other peptidases that lead to even more circulating proteolytic metabolites of CXCL12 (Richter et al., 2014). Until recently DPP4-cleaved CXCL12 has generally been thought to be inactive, or at least much less active than intact CXCL12 (Anderluh et al., 2016; Crump et al., 1997; Kato et al., 1998).

The two main receptors for CXCL12 are CXCR4, a ligand concentration biased G-protein coupled receptor (GPCR), and CXCR7 (AKA - Atypical Chemokine Receptor 3 or ACKR3), a β-arrestin associated receptor that has a 1000-fold higher affinity for CXCL12 than CXCR4 (Quinn, Mackie, & Caron, 2018; Reid et al., 2018; Sanchez-Martin, Sanchez-Mateos, & Cabanas, 2012). Data suggests that both receptors can homodimerize, as well as heterodimerize with each other, or with other receptors including α1A/B-adrenoceptors (Decaillot et al., 2011; Levoye, Balabanian, Baleux, Bachelerie, & Lagane, 2009; Tripathi et al., 2015). This ability to dimerize regulates receptor functions and affects their downstream signaling events (Ferre et al., 2014; Milligan, Canals, Pediani, Ellis, & Lopez-Gimenez, 2007; Rozenfeld & Devi, 2010; Tripathi et al., 2015). ACKR3 may have additional heterodimerization partners including epidermal growth factor receptor (Salazar et al., 2014). The potential for multiple heterodimeric complexes with CXCL12 receptors opens the door for possible combinations that respond to DPP4, and other N-terminal, cleaved CXCL12 isoforms. The ligand, CXCL12, can itself homodimerize, and it can activate the CXCR4 receptor either in monomeric or a dimeric form (Drury et al., 2011; Roy et al., 2015). There is, however, a ligand bias associated with CXCR4 response to monomeric or dimeric CXCL12 activation. In this case, monomeric CXCL12 activation of CXCR4 leads to G-protein-dependent signaling. However, as the CXCL12 ligand concentrations increase, the likelihood of both ligand binding pockets of the CXCR4 receptor dimer being occupied increases as well. This is accompanied with a switch from GPCR-mediated signaling to β-arrestin-dependent signaling, which blocks actin remodeling and inhibits cell migration (Drury et al., 2011; Ziarek et al., 2017).

7.1. Receptor activation by DPP4-cleaved CXCL12

Crump et al., assessed the ability of N-terminal modified, and other CXCL12 variants, to bind to and activate CXCR4 demonstrating that removal of the first 2 amino acids or beyond dramatically inhibited or eliminated binding to and activation of CXCR4 (Crump et al., 1997). More recently, Janssens et al., used CXCR4-transfected, ACKR3-negative CHO and COS-7 cells to test receptor activation and signaling specifically through the CXCR4 receptor. It was found that starting at 100 pM, intact CXCL12(1-68) induced IP3 accumulation in the cytosol, and triggered phosphorylation of the second messengers ERK and Akt, in a dose-dependent manner. In contrast DPP4-cleaved CXCL12(3-68) even at concentrations up to 100 nM, did not induce IP3 accumulation, or ERK and Akt phosphorylation (Janssens et al., 2017). Janssens et al., also demonstrated that while DPP4-cleaved CXCL12α cannot activate CXCR4, it can still recruit β-arrestin through activation of the ACKR3 receptor. However, the activation of ACKR3 is about 10-fold less than that induced by intact CXCL12 (Janssens et al., 2017) (Table 8). Cheng et al., showed very similar results using the Presto-Tango β-arrestin 2 recruitment assays for CXCL12(1-68) and CXCL12(3-68). The assays showed that while 1 μM CXCL12(1-68) recruited β-arrestin 2 through both CXCR4 and ACKR3, CXCL12(3-68) only managed to recruit β-arrestin 2 through the CXCR4 receptor at extremely high doses in vitro [EC$_{50}$ (nM) >10^3] with no detectable efficiency% while the ACKR3 receptor binding of CXCL12 (3-68) lead to ACKR3 recruitment of β-arrestin 2 that was significantly greater [EC$_{50}$ (nM) >13.3±13.5], but reduced to about 20% efficiency compared to intact CXCL12(1-68) (Cheng, Eby, et al., 2017). Ziarek et al., 2017 also reported that DPP4-truncated CXCL12(3-68) shows a dramatic drop in CXCR4 binding affinity, in addition to complete loss of G protein agonist activity. They also described that cleaved CXCL12 possesses a very weak CXCR4 GPCR antagonistic activity (Ziarek et al., 2017).

Research done by Szpakowska et al., on CXCL11, another cytokine that activates CXCR3 and ACKR3, and is cleaved by DPP4 in a fashion similar to CXCL12, showed that DPP4-cleaved CXCL11 loses its ability to activate the CXCR3 receptor, but retains the ability to bind and activate ACKR3 (Szpakowska et al., 2018). This result, along with the previously mentioned CXCL12 receptor binding and activation assays, shows that removal of N-terminal lysine/proline by DPP4 is not able to initiate GPCR-biased activity through CXCR4, but can still induce β-arrestin mediated activity through ACKR3 and possibly to a lesser extent through CXCR4 β-arrestin mediated activity.

7.2. CXCL12 GAG binding affinity

As mentioned previously, CXCL12 concentration and biological activity are affected by binding to GAGs. Janssens et al., utilized heparan

Table 8
Effect of N-terminal cleavage on receptor activation and biological functions of CXCL12α and β

	Intact CXCL12	Cleaved CXCL12	Ref.
CXCR4 receptor (G-protein agonist)	High	No activation	Cheng, Eby, et al. (2017), Janssens et al. (2017), Ziarek et al. (2017)
CXCR4 receptor (β-arrestin recruitment)	High (concentration biased)	Very low with EC$_{50}$ >10^3	Cheng, Eby, et al. (2017)
ACKR3 receptor (β-arrestin recruitment)	High	Decreased by 80-90%	Cheng, Eby, et al. (2017), Janssens et al. (2017)
GAG binding	High	Slightly decreased	Janssens et al. (2017)
BMSCs osteogenic differentiation	increases	inhibits	Elmansi et al. (2018)
Cell migration	chemotactic	inhibits	Elmansi et al. (2018), Janssens et al. (2017), Kato et al. (1998)
Anti-HIV-1 activity	Present	Absent	Kato et al. (1998)

sulfate and dermatan sulfate coated plates to compare the binding affinity of intact and cleaved CXCL12. The results showed a moderate, but significant, decrease in affinity of cleaved CXCL12 to dermatan sulfate compared to intact CXCL12. However, there was no significant difference in affinity of both intact and cleaved CXCL12 to heparan sulfate, which is thought to be a major sink for extracellular CXCL12 (Janssens et al., 2017).

7.3. CXCL12 effect on bone marrow mesenchymal stem cells differentiation

The role of CXCL12 in bone health is well established. CXCL12β was previously shown to potentiate the osteoinductive effect of bone morphogenetic protein 2. This effect was shown *in vitro* in genetically engineered bone marrow-derived mesenchymal stem cells (BMSCs), and *in vivo* in bone regeneration in critical-size rat calvarial defects (Herberg et al., 2014; Herberg et al., 2015). Our lab recently found that while intact CXCL12β(1-72) induces osteogenic differentiation in BMSCs, cleaved CXCL12β(3-72) inhibits osteogenic differentiation (Elmansi et al., 2018) (Table 8). This result has major implications, as the balance between osteogenic and adipogenic differentiation in BMSCs is a crucial aspect in bone healing and healthy bone aging (Infante & Rodriguez, 2018).

7.4. CXCL12 effect on cell migration

CXCR4 is required for CXCL12-mediated chemotaxis, and is a co-receptor for HIV cell binding and transfection. A paper by Shioda et al., in 1998 demonstrated that DPP4 abolished both chemotactic and anti-HIV-1 activities for CXCL12α and CXCL12β. Shioda et al., infected two different cell lines with a recombinant CXCL12α-expressing vector. The two cell lines were H9, a DPP4-expressing T-cell line, and MT4, a T-cell line that does not express DPP4. After 3 days, the results they found were intriguing; western blot analysis showed while there were similar total levels of CXCL12α in the supernatant of both cell lines, the chemotactic and anti-HIV-1 activities widely varied from remarkably high levels in non-DPP4 expressing MT4 cells to very low activity in DPP4 expressing H9 cells (Kato et al., 1998). Although this experiment did not directly test the biological activity of DPP4-cleaved CXCL12, it hints that there is an antagonistic action resulting from having DPP4 in close proximity to released CXCL12.

Another experiment by Janssens et al., showed that DPP4-cleaved CXCL12α does not induce endothelial cell migration compared to intact CXCL12α. However, there was no control group to test the possibility that cleaved CXCL12α inhibits migration, instead of simply being inactive (Janssens et al., 2017). Christopherson et al, reported that DPP4-cleaved CXCL12α(3-68) does not induced the migration of CD34^{+} HSCPs and even inhibits cellular migratory activity induced by intact CXCL12α (1-68). They suggested that DPP4 truncation of CXCL12α could represent a novel mechanism that regulates migration, mobilization, and homing of HSPCs (Christopherson, Cooper, & Broxmeyer, 2003; Christopherson II, Hangoc, & Broxmeyer, 2002; Christopherson II, Hangoc, Mantel, & Broxmeyer, 2004). Moreover, our lab recently found that pretreating BMSCs with cleaved CXCL12β(3-72) for 16 hours, diminishes their ability to migrate towards media containing intact CXCL12β(1-72) in transwell assays. This result is important in understanding stem cell migration and cancer cell metastasis. It also means that DPP4-cleaved CXCL12 is not simply inactive, but possesses a separate bioactivity that might be antagonistic to intact CXCL12 (Elmansi et al., 2018) (Table 8) (See Fig. 6). However, since DPP4 cleaved CXCL12 does not appear to effectively bind to CXCR4 based on competitive displacement of radiolabeled CXCL12 (1-68) by cold CXCL12 (3-68) [CXCL12(3-68) $k_d = 464 \pm 2nM$ compared to Kd = 1.44 ± 1.5 nM for CXCL12(1-68) (Ziarek et al., 2017) the reports of apparent antagonistic activity actually may be mediated by ACKR3 or alternative mechanisms via interactions with other receptors (Cheng, Eby, et al., 2017; Janssens et al., 2017). For example, ACKR3 activation may down-regulate CXCR4 expression and therefore G-protein coupled signaling directly, or as a consequence of CXCR4/ACKR3 heterodimerization, which results in β-arrestin signaling while abrogating G-protein coupled signaling through CXCR4. Therefore, ACKR3 activation leads to decreased CXCR4 signaling and inhibition of CXCR4 mediated migration and other actions (Decaillot et al., 2011; Janssens, Struyf, & Proost, 2018; Levoye et al., 2009; Zabel et al., 2009). (See Fig. 6.)
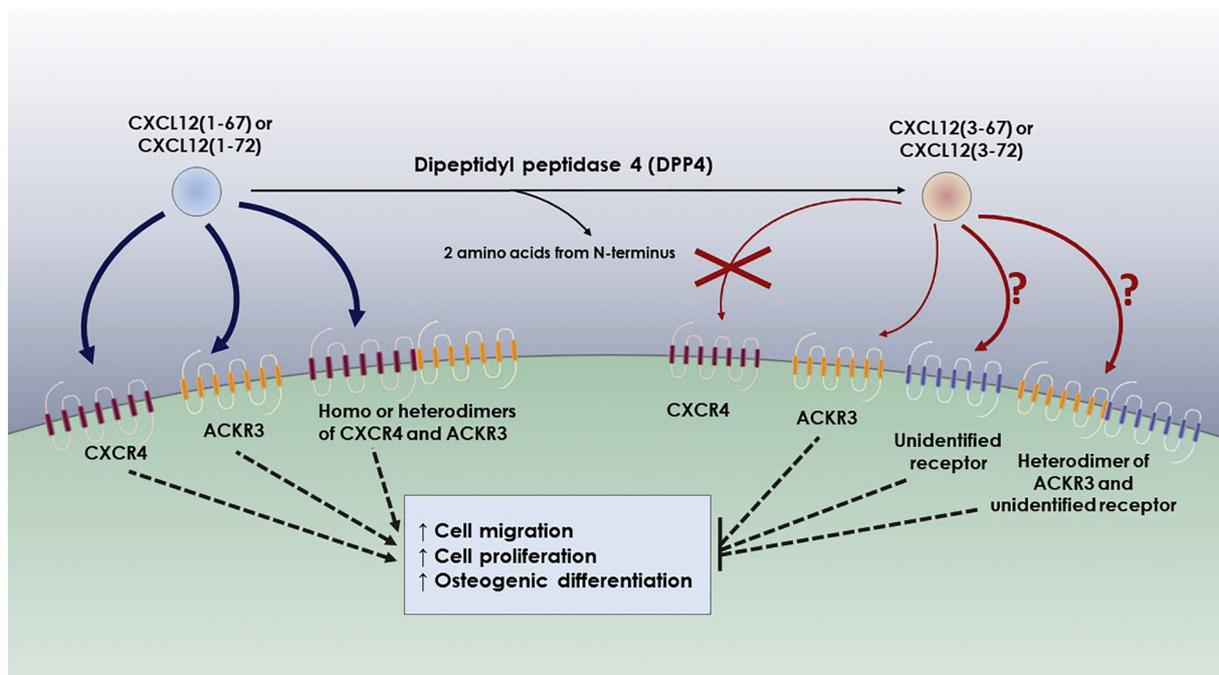

Fig. 6. Effect of DPP4 proteolysis on receptor activation and biological functions of CXCL12

7.5. CXCL12 effect on cell viability

We previously showed that intact CXCL12β mediates cell survival via upregulation of the autophagic pathway and inhibition of apoptosis in BMSCs (Herberg, Shi, et al., 2013). However, another form of cleaved CXCL12(5-67) produced by the action of matrix metalloproteinase-2, was shown to induce NSC death through activating the intrinsic apoptotic pathway. This was evident by increased caspase 3/7 activation in NSCs treated with cleaved CXCL12(5-67) compared to control. Intact CXCL12 on the other hand was shown to inhibit apoptosis and decrease caspase 3/7 activation in NSCs compared to control (Adelita, Stilhano, Han, Justo, & Porcionatto, 2017).

7.6. CXCL12 effect on brain and nervous system

While there is no available data on DPP4-cleaved CXCL12(3-67) activity in the brain, studies showed that the previously-mentioned cleaved CXCL12 (5-67) is neurotoxic to human and mouse neurons with no chemotactic properties. It was also found that higher levels of CXCL12(5-67) in brain were associated with HIV-1 infection (McQuibban et al., 2001; Zhang et al., 2003). The interesting and relevant part, is that this form of cleaved CXCL12(5-67) did not act through CXCR4, the regular receptor for intact CXCL12, but exerted its neurotoxic and immunogenic actions through the chemokine receptor CXCR3, which also shares the ligand CXCL11 with ACKR3, which is promiscuous in being activation-prone by N-terminal modified CXCL11 and CXCL12 (Benhadjeba, Edjekouane, Sauve, Carmona, & Tremblay, 2018; Vergote et al., 2006). This does not mean that DPP4-cleaved CXCL12 acts through CXCR3, but it reinforces the idea that a proteolytic enzyme can generate a new cryptic ligand to a receptor that was not activated by the intact parent peptide.

7.7. CXCR4 and DPP4 interactions

Among the non-substrate proteins that bind CD26 are a number of receptors including the CXCL12 receptor CXCR4 (Havre et al., 2008). Binding of CXCL12 initiates the internalization of both CXCR4 and CD26 in the T-cell line, Jurkat J32, the B-cell line, SKW6.4, and peripheral blood lymphocytes, suggesting that these two proteins may function together and emphasizing the importance of understanding the actions of DPP4 cleaved ligands (Herrera et al., 2001).

7.8. CXCL12 summary

CXCL12 is one of the most thoroughly studied cytokines in the literature. However, data regarding biological functions of DPP4-cleaved CXCL12 are just beginning to emerge. There have been a number of papers measuring DPP4-cleaved CXCL12(3-68) affinity, binding, and potential to activate CXCR4 or ACKR3 receptors (Cheng, Eby, et al., 2017; Janssens et al., 2017). It has been shown that DPP4-cleaved CXCL12 (3-68) can bind and activate ACKR3 with reduced affinity, while it cannot activate CXCR4 at all, at least via GPCR signaling. There is however a suggestion that CXCL12(3-68) may signal via β-Arrestin 2 pathways for both ACKR3, and possibly CXCR4 (Cheng, Eby, et al., 2017; Janssens et al., 2017; Ziarek et al., 2017). In terms of binding to GAGs in extracellular matrix, cleaved CXCL12(3-68) binds a subset of GAGs with a slightly decreased affinity compared to intact CXCL12. The main biological function measured for cleaved CXCL12 in the literature has been its inability to provide the anti-HIV activity of the intact isoform (Crump et al., 1997; Proost et al., 1998). Our lab recently found that DPP4-cleaved CXCL12β(3-72) has an antagonistic-like action to intact CXCL12β(1-72) in terms of BMSCs cell migration and osteogenic differentiation (Elmansi et al., 2018), while intact CXCL12β(1-72) induces BMSCs migration and osteogenic differentiation, cleaved CXCL12β(3-72) inhibits both of those CXCR4 G-protein mediated functions (Elmansi et al., 2018). Again, this likely does not represent direct competitive inhibition of the intact isoform with the CXCR4 receptor, certainly not through the CXCR4 GPCR mechanism, and it is unclear if CXCL12α(3-68) and CXCL12β(3-72) can bind effectively at physiological levels to CXCR4 (Cheng, Eby, et al., 2017; Crump et al., 1997; Janssens et al., 2017; Ziarek et al., 2017). Further work needs to be done to assess other biological functions and potential mechanistic pathways through which DPP4-cleaved CXCL12 is acting.

8. Conclusion

DPP4 proteolytic cleavage of different peptide hormones does not simply deactivate them. Rather, many studies have shown that DPP4 cleavage leads to changes in the intact ligand/substrate's biological function mediated through alterations in the normal receptor (s) interactions or revealed cryptic interactions with receptors that do not recognize the intact ligand. Additionally, biological functions and receptor interactions usually attributed to the intact ligand may actually be due to the DPP4-cleaved ligand. While this review focuses on the consequences of DPP4 enzymatic cleavage of five different substrates that belong to three different classes of biological molecules, it is important to mention that these constitute only a small number of DPP4 substrates. A large array of other proteins –including cytokines, modulatory factors, colony-stimulating factors, etc.- have been reported to be cleaved by DPP4 in different tissues and organs (Broxmeyer et al., 2012; Christopherson, Cooper, Hangoc, & Broxmeyer, 2003; Ou et al., 2013).

In the case of GLP-1, it was found that some of the actions attributed to intact GLP-1 can be attenuated or even completely blocked upon inhibition of DPP4 proteolytic activity. This, in addition to the previously mentioned findings, suggests that cleaved GLP-1 is itself responsible for some of the physiological actions that has been attributed to intact GLP-1. Blocking of GLP-1R reverses intact GLP-1 activity, with no effect on cleaved GLP-1 activity. This observation indicates that cleaved GLP-1 is also acting through an unidentified receptor that is distinct from GLP-1R. This surprising conclusion about DPP4-cleaved ligands is paradigm shift that reveals a critical gap in our understanding and interpretation of research and clinical data related to vital signaling pathways. In the case of GLP-1 the clinical implications are significant, since it affects our understanding of insulin regulation in general and specifically through new implications of how anti-diabetic DPP4 inhibitors work. Further research in this area is essential, to better understand the potentially beneficial biological actions of cleaved GLP-1 that can be suppressed by the use of DPP4 inhibitors, as well as better understanding the physiology of extending the functional half-life of intact GLP-1.

For GIP, DPP4 cleavage introduces a GIP receptor antagonistic feature that is strengthened by C-terminal cleavage by other proteolytic enzymes. While for the two neuropeptides PYY and NPY, DPP4 cleavage means the production of a more selective Y2 receptor agonist, instead of the non-selective intact form.

For CXCL12, it was found that DPP4 cleavage completely abolishes its ability to initiate CXCR4 GPCR activation, while it retains some of its ability to activate ACKR3, making it more selective to β-arrestin signal transduction pathways instead of GPCR pathways. Further, the ability of DPP4-cleaved CXCL12 to activate ACKR3 inhibiting CXCR4 signaling might explain DPP4-cleaved CXCL12's apparent CXCR4 antagonistic action in light of its apparent inability to bind effectively to CXCR4 (Ziarek et al., 2017). Although this last point is needs further clarification. Importantly, there are hints that DPP4-cleaved CXCL12 may also act through alternative receptors and signaling pathways. This underscores the need to explore DPP4 cryptic ligands bioactivity not only for CXCL12, but more broadly among DPP4 substrates. In addition to the clinical significance these findings carry, they can also help us predict or understand how DPP4 and DPP4 inhibitors may affect cellular functions and biological processes of other important chemokines and polypeptides.

Further, this review highlights the crucial need to standardize the collection and processing of samples to preserve the true *in vivo* molecular profiles. This is both in terms of the total quantities of specific molecules as well as the ratio or individual profiles of different proteolytic metabolites. Additionally, there is a critical need to develop new tools to measure these ligand isoforms for accurate quantitation and interpretation of experimental results. Importantly, we are also proposing that plasma, serum, urine, and other biological samples collected in clinical trials and for biorepositories need to have the samples protected at the point of collection from DPP4 and other proteolytic or enzymatic degradation to allow for not only accurate prospective analysis, but for retrospective use of these tissue banks and biorepositories. This has real world implications in understanding normal and pathological systems and quality of life and even life-or-death repercussions in the development of therapeutics for major diseases such as diabetes, osteoporosis, inflammation, stroke, myocardial infarction, wound repair and cancer.

Conflict of interest statement

The above mentioned funding did not lead to any conflict of interests regarding the publication of this manuscript. The authors also declare that there is no other conflict of interest regarding the publication of this manuscript.

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