The Multifunctional Post-proline Dipeptidyl Peptidase, DPP9, in Mice, Cell Biology and Immunity

Margaret G. Gall and Mark D. Gorrell

Abstract

Dipeptidyl peptidase 9 (DPP9) is a ubiquitous intracellular post-proline protease of the DPP4 (S9b) family of atypical serine proteases. Emerging data support roles for DPP9 in intracellular signalling, particularly in the epidermal growth factor receptor pathway, in immune cells, particularly in macrophages and antigen processing, and in energy metabolism. The focus of this review is the roles of DPP9 in regulating physiological and cellular processes. Such data is derived from a genetically modified mouse strain and from manipulations of cell lines. The mouse strain that lacks DPP9 enzyme activity is homozygous lethal. DPP9 alters behaviours, such as cell adhesion, of cancer cell lines. This review points to the functional importance of DPP9 in immunity, metabolism and cancer.

Keywords

Dipeptidyl peptidase · Mouse models · Neonate development
Fibroblast activation protein

Abbreviations

AMC	Amino methylcoumarin
AMPK	AMP-activated protein kinase
B-CLL	B-cell chronic lymphocytic leukaemia
CAF	Cancer-associated fibroblasts
CXCL	CXC chemokine ligand

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DEN	Diethylnitrosamine
DPP	Dipeptidyl peptidase
DSS	Dextran sulphate sodium
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ESFT	Ewing sarcoma family of tumours
FAP	Fibroblast activation protein
FGF	Fibroblast growth factor
HCC	Hepatocellular carcinoma
HFD	High fat diet
HSC	Hepatic stellate cells
IRS	Insulin receptor substrate
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
MMP	Matrix metalloproteinase
NEM	N-ethylmaleimide
NPY	Neuropeptide Y
PARP	Poly(ADP-ribose) polymerase
PGC-1α	PPAR coactivator-1α
PBMC	Peripheral blood mononuclear cells
pNA	P-nitroaniline
POP	Prolyl oligopeptidase
PPAR	Peroxisome proliferator-activated receptor
SUMO	Small ubiquitin-like modifier
TAA	Thioacetamide
TAM	Tumour-associated macrophages
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor α
VEGF-A	Vascular endothelial growth factor-A
WT	Wild-type

1 Introduction

1.1 The Dipeptidyl Peptidase 4 (DPP4) Enzyme Family

The DPP4 family of proteases has important roles in regulating physiological and cellular processes. The ubiquitous DPP4 family is the S9b protease subfamily of the prolyl oligopeptidases [1] of atypical serine proteases that contain a conserved catalytic triad of serine, aspartate and histidine [2]. Prolyl endopeptidase (PEP) and the DPP4 family enzymes possess their ability to hydrolyse a post-proline peptide bond. The DPP4 family enzymes can cut two residues from the N terminus of a protein substrate [3–5]. The presence of a proline near the N terminus of a peptide (Fig. 1) generally confers resistance against protease degradation and occurs in many different biologically active peptides, including chemokines, incretins and neuropeptides. Thus, the specialised ability of dipeptidyl peptidases to cleave the post-proline bond is useful to degrade such peptides [6].

The DPP4 protein family consists of six members: four enzymatic members and two non-enzymatic members. DPP4, DPP8, DPP9 and FAP have DPP activity, while FAP is also a post-proline endopeptidase [3–5, 7]. The two non-enzymatic members are DPP6 (DPP-X) and DPP10, also known as dipeptidyl peptidase like (DPL) protein-1 and DPL-2 [8–10] (Fig. 2).

1.1.1 DPP4

DPP4 is the prototypical and the most well-characterised member of the family. It displays enzymatic activity in both the dimeric cell surface membrane-bound form and the soluble circulating form [12, 13]. DPP4 is expressed by epithelial cells in the liver, gut, uterus and kidney, by immune organs, by capillary endothelium [14],

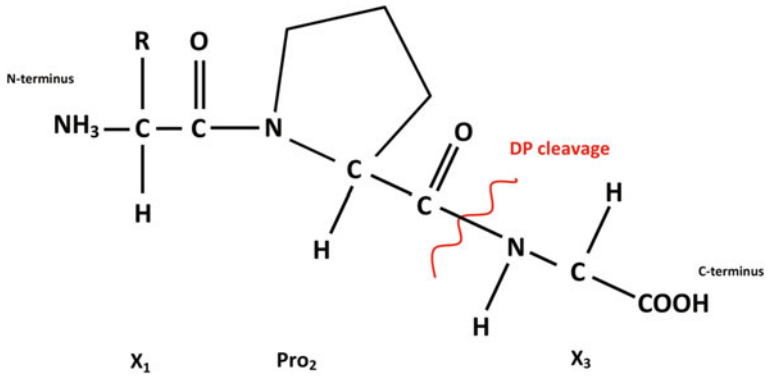

Fig. 1 Cleavage of a post-proline bond. A tri-peptide containing a proline at position two creates a bend in the peptide chain. Proline-containing peptides pose a specific problem for proteases due to structural constraints imposed by the pyrrolidine ring that prevents hydrolysis by most peptidases. Dipeptidyl peptidase cleavage by DPP4, DPP8 and DPP9 occurs C-terminal to the proline residue

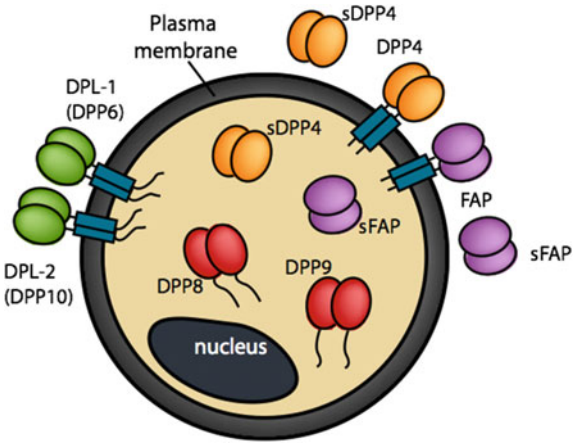

Fig. 2 Cellular localisation of the six members of the DPP4 protein family. DPP4 and FAP are type-II proteins that are membrane-bound cell surface proteases and also have soluble forms. DPP8 and DPP9 are intracellular proteases. DPP6 and DPP10 are membrane-bound, non-enzymatic proteins. Adapted from [11]

and by acinar cells of mucous and salivary glands and pancreas [15]. The soluble form is present in serum, seminal fluid, saliva, kidney, liver and bile [12, 15–18].

Functionally, DPP4 is involved in numerous processes throughout the body, including immunological, haematological, endocrine and metabolic. These include the ability to participate in chemokine inactivation and also to have a role in apoptosis, lymphocyte activation and cell migration, along with the capacity to cleave a wide range of small bioactive proteins [16, 19–22]. Most noted of these is the ability of DPP4 to rapidly inactivate the incretins glucagon-like peptide-1 (GLP-1) and glucose insulinotropic peptide (GIP) [23] leading to the inhibition of DPP4 as a successful type 2 diabetes therapy. DPP4 is also known as CD26 and, as such, has roles in the immune system in T cell activation and proliferation and T helper 1 responses to foreign antigens. Cell surface expression on T cells greatly increases following stimulation with antigen or mitogens [15, 17, 24].

1.1.2 FAP

FAP, which is also known as seprase, has 52% amino acid sequence identity with DPP4. However, FAP possesses both dipeptidyl peptidase and endopeptidase activities, which enables it to cleave a post-proline bond two or more amino acids from the N terminus of a protein (Fig. 3) [7].

While DPP4 is abundant in most tissues, FAP is at very low levels [18] with high-level expression being limited to sites of tissue remodelling and areas of stromal activation, such as in solid tumours, wound healing, tissue damage and inflammation [25, 26], during mouse embryogenesis [27] and in activated hepatic stellate cells in cirrhotic liver [28–30]. FAP has been isolated in a truncated soluble form from human plasma and serum [31, 32]. FAP activity levels in normal and

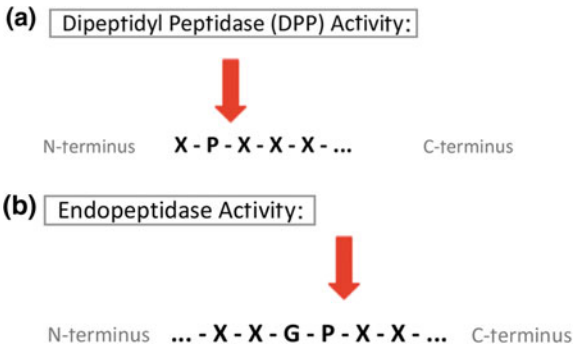

Fig. 3 Enzyme activity of FAP. **a** Dipeptidyl peptidase activity involves the cleavage of two amino acids of the N terminus of a protein following a proline residue. **b** Endopeptidase activity involves the cleavage of a post-proline bond that is more than two amino acids from the N terminus of a protein

diseased tissue from humans, mice and baboons have been measured using the novel FAP-specific substrate, 3144-AMC. In mice, uterus, pancreas, submaxillary gland and skin showed the greatest levels of FAP activity [18].

While FAP has been shown to cleave some DPP4 substrates in vitro [33], until recently there were no known physiological substrates of FAP's dipeptidyl peptidase activity. The first study to characterise the physiological DPP substrate repertoire of endogenous FAP in mammalian plasma showed a potential function of FAP in neuropeptide signalling in liver and liver cancer [34]. In addition to its dipeptidyl peptidase activity, FAP also has endopeptidase activity cleaving α2-antiplasmin [31] and denatured type I collagen [35]. The gelatinase activity of FAP most likely contributes to extracellular matrix degradation.

Because of its pattern of expression by activated stromal fibroblasts in many cancers, FAP has been studied as a potential therapeutic target in tumours. FAP roles in cancer biology have been reviewed previously [36–38]. Strategies for targeting FAP for cancer therapy and the role of FAP seem to be very contextual, so the expression pattern and substrates of FAP require greater definition to better predict the effects of targeting this protease.

1.1.3 DPP6 and DPP10

DPP6 and DPP10 have 53% amino acid similarity with each other and are highly homologous to DPP4 with sequence identities of 33 and 32%, respectively [5, 10]. They both form dimers but lack the catalytic serine and a nearby tryptophan that are essential for enzymatic activity. DPP6 has a wide tissue distribution, but DPP10, in contrast, has expression limited to human brain, adrenal gland and pancreas [5, 10, 39]. The functional effects of these proteins appear to be through binding interactions, and there is evidence of an association with neuronal diseases and asthma [10, 40, 41].

1.1.4 DPP8 and DPP9

DPP8 and its closest relative DPP9 are the most recently discovered members of the DPP4 gene family [4, 5, 42, 43]. As DPP4, DPP8 and DPP9 all have DPP4-like enzymatic activity and ubiquitous expression, the discovery of DPP8 and DPP9 has called for the reinterpretation of previous DPP4 data [4, 42]. This was necessary to determine which functions had been attributed to DPP4 that may instead be accounted for by DPP8 and DPP9.

Unlike DPP4, DPP8 and DPP9 are intracellular proteins and are therefore likely to have different biological roles. As there are currently no substrates or inhibitors that distinguish DPP8 from DPP9, their characteristics are usually outlined together but this review will focus upon DPP9. DPP9 is currently under study in many fields including cell biology, immune-biology and tumour biology [44].

2 Structure of DPP8 and DPP9

DPP8 and *DPP9* are located on human chromosomes 15q22 and 19p13.3 [42, 43] and mouse chromosomes 9 and 17, respectively. In *DPP4* and *FAP*, the sequence adjacent to the active-site serine is encoded by two exons while the homologous region in *DPP8* and *DPP9* is encoded by a single exon, which suggests that the *DPP8* and *DPP9* genes arose at an earlier evolutionary stage and may have arisen by gene duplication [43]. The amino acid homology between human and mouse DPP8 and DPP9 is 95 and 92% identity, respectively [4, 43]. No crystal structures of DPP8 or DPP9 exist, but homology models have been built using the known structures of DPP4, DPP6 and FAP [45–47]. In these models, DPP9 is depicted with an α/β-hydrolase domain containing a Ser-Asp-His catalytic triad and an 8-blade β-propeller domain containing two glutamic acids essential to functionality of the catalytic pocket (Fig. 4).

While structurally similar to DPP4 and FAP, DPP8 and DPP9 are longer at the N terminus but lack a transmembrane domain and are intracellular proteins [4, 42]. Another structural difference between DPP4 and DPP9 is that selective point mutations in the C-terminal loop can inactivate DPP9 while maintaining the dimeric structure [48]. This confirms the importance of the C-terminal loop for DPP8 and DPP9 enzymatic function. Finally, since DPP8 has been shown to have a larger substrate pocket than DPP4 [47], DPP8 and DPP9 might have a larger β-propeller domain and/or may contain a separate element of tertiary structure at the N terminus of the protein.

Fig. 4 A model of DPP9 protein structure. DPP9 residues 51–863 are depicted in ribbon representation of the monomer. The α/β-hydrolase domain is in *red* and the 8-blade β-propeller domain in *green*. The catalytic triad (Ser-Asp-His) is shown as *blue* spheres in the hydrolase domain and 2 glutamates essential for catalysis as *yellow* spheres in the propeller domain. An extended arm (^{285}VEVIHVPSPALEERKTDSYR^{304}) in the propeller surface of DPP9 that is critical in SUMO1–DPP9 interaction is coloured *pink*. Modified from [44, 45]

3 Expression Profiles and Tissue Distribution of DPP8 and DPP9

Both DPP8 and DPP9 have isoforms. Human DPP8 has several mRNA transcripts identified: one is abundant in testis, prostate and muscle [5, 42], while another longer transcript variant has intense signals in adult testis [49]. Bioinformatics analysis of NCBI database sequences has identified at least two other isoforms [50].

Two forms of DPP9 cDNA have been cloned, with the short form (DPP9-S) encoding 863 amino acids [4, 43] and the long form (DPP9-L) encoding 971 amino acids [4]. DPP9-S is ubiquitously expressed including high levels in liver, heart and skeletal muscle, while DPP9-L is much less abundant and is predominantly expressed in skeletal muscle. The N-terminal extension of DPP9-L contains a nuclear localisation signal (NLS) allowing it to enter the nucleus [51]. Endogenous nuclear DPP9 has been visualised in mouse fibroblasts [52].

In canine and porcine small intestine, lung, kidney and pancreas, differential relative abundance of DPP8 and DPP9 has been measured by RT-PCR analysis with DPP9 having the greater expression [53]. In adult mice, greater numbers of DPP9 transcripts are in brain, skin, colon and thymus compared with DPP4 [53, 54].

DPP8 mRNA is upregulated in human adult testis compared to foetal testis [49]. DPP8 and DPP9 is in spermatozoids embedded in the epithelium of the seminiferous tubules [55], while the mRNA is in spermatogonia and spermatids [56]. DPP9-S has been purified from bovine testes [57]. Taken together, these studies suggest a role for DPP8 and DPP9 in spermatogenesis.

Using in situ hybridisation, immunohistochemistry and enzyme assays on baboon, mouse and human tissues, the ubiquitous expression and distribution of DPP8 and DPP9 have been visualised in lymphocytes and epithelial cells in the gastrointestinal tract, liver, lung, spleen, lymph node and skin, as well as in pancreatic acinar cells, muscle, adrenal gland, testis and Purkinje cells and the granular layer of the cerebellum [56]. These comprehensive findings concord with related studies [55, 58, 59].

Analyses of mRNA and protein levels in Sprague Dawley rat and cynomolgus monkey showed similar ubiquitous expression and tissue distribution correlated with DPP8/9 enzymatic activity and also detected the DPP8 and DPP9 proteins in some capillary endothelia [14]. DPP8 and DPP9 have been shown to be in rat primary endothelial cells of aortic, endocardial and cardiac microvascular origin [60] with a greater abundance of DPP8 protein over DPP9. However, in human carotid artery endothelial cells, DPP9 has been the only DPP4-like enzyme detected using immunohistochemistry, suggesting a regulated expression of DPPs in endothelial cells.

Both DPP8 and DPP9 occur in tumours [4, 56, 61], hepatocytes [56, 62, 63], macrophages [64] and lymphocytes [56, 63, 65] but are absent from activated stellate cells [56].

Recent reviews provide more detailed information regarding the expression and distribution of DPP8 and DPP9 [19, 22, 44, 66].

4 Enzymatic Activity and Substrate Specificity of DPP8 and DPP9

Although originally thought to be monomers, both DPP8 and DPP9 are, like DPP4, catalytically active as dimers [48, 67, 68]. Both DPP8 and DPP9 have optimum activity at neutral pH of 7.0–8.5 [5, 42, 48], which is consistent with intracellular localisation. DPP8 and DPP9 have reversible changes in enzymatic activity intensity, dependent on the redox state of their cysteine moieties, with activity decreased by oxidation and increased by reduction [45, 69]. DPP9 is allosterically activated by SUMO1 [70]; however, the small peptide region of SUMO1 that interacts with DPP9 is also a non-competitive inhibitor of DPP9 activity with inhibition dependent on the DPP9 arm motif (Fig. 4) [71].

Both enzymes have similar substrate specificity, preferring a proline in the penultimate P1 position and small or basic amino acid residues at the P2 position of synthetic substrates [48, 68, 69]. Similarly, preferred natural substrates have a P1 proline preceded by an alanine in the P2 position [72], but alanine can be in P1

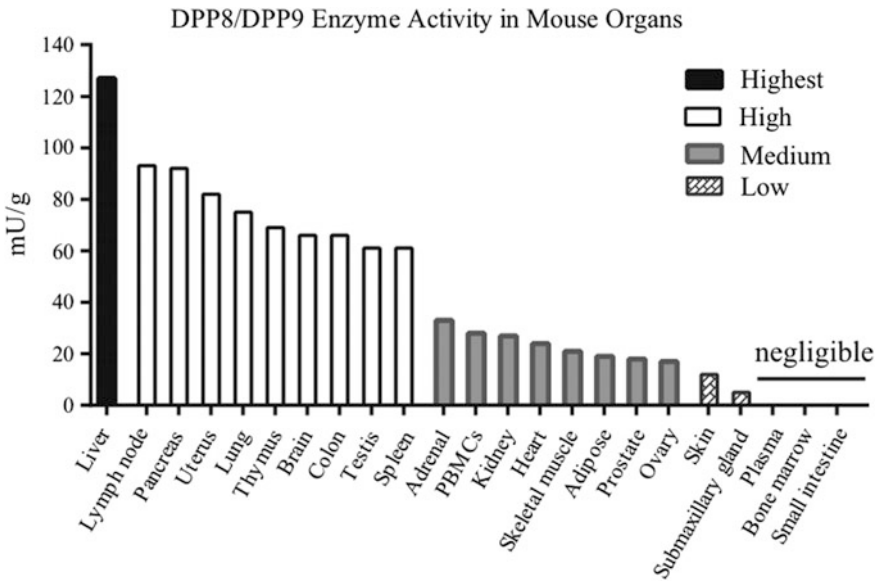

Fig. 5 DPP8/DPP9 enzyme activity in mouse organs. The approximate intensity of DPP8/9 enzyme activity was measured by Yu and colleagues [56] from mouse organs in DPP4-gene knockout mice with/without the addition of the DPP8/9 inhibitor NEM (N-ethylmaleimide). NEM-inhibited activity was subtracted from total DPP activity to estimate the activity derived from DPP8 and DPP9. Modified from [44]

[67, 73]. Ala-Pro- and Gly-Pro-containing fluorogenic and chromogenic substrates have been routinely used to detect DPP8/9 enzymatic activity (Fig. 5), but H-Arg-Pro-pNA is also hydrolysed [4, 56]. We recently showed that DPP9 is much more efficient than DPP8 at cutting after alanine in P1 [73].

DPP8 and DPP9 are able to cleave a number of naturally occurring extracellular peptides and chemokines in vitro but with reduced rates of hydrolysis compared to DPP4 [67, 73, 74]. Whether extracellular substrates of these intracellular DPPs have physiological relevance is unclear. It has previously been shown that neuropeptide Y (NPY) can be cleaved by DPP4, DPP8, DPP9 and FAP [33, 34, 67, 75, 76]. NPY extracted from rat brain can be cleaved in the presence of a DPP4 inhibitor, suggesting that DPP8 and DPP9 can cleave NPY in vivo [76]. Moreover, NPY is a substrate for DPP8 and DPP9 in intact cells, not just cell extracts, and in doing so regulates Ewing sarcoma family tumour (ESFT) cells by reducing NPY-induced cell death [77].

A role of DPP9 in antigen presentation and peptide turnover was first shown by the identification of the antigenic peptide $RU1_{34–42}$ as its first natural substrate. Silencing of DPP8 and DPP9 using siRNA resulted in increased RU1 presentation only in DPP9-silenced cells, which indicated that the $RU1_{34–42}$ antigen is an in vivo substrate of DPP9 [69].

DPP9 substrates have been sought using a degradomic approach and resulted in the identification and validation in vitro of numerous potential substrates, with adenylate kinase 2 and calreticulin confirmed as DPP8 and DPP9 substrates. Those substrates suggested a role for DPP8 and DPP9 in cellular metabolism and homoeostasis [72].

Most recently, two-dimensional differential in-gel electrophoresis (DIGE) was applied to cytoplasmic and nuclear extracts of mouse cells lacking endogenous DPP9 activity (the DPP9-GKI mouse, see below) to identify novel DPP9 substrates [73], with nine being confirmed as substrates by MALDI-TOF or immunoblotting. This study also identified a DPP9-specific consensus site for cleavage that was not recognised by DPP8, suggesting that these two proteases have different in vivo roles. The substrates included two key immune regulators, CXCL10 and IL1RA, and the putative DPP9 substrates S100-A10, SET and NUCB1, which are mediators of immunity and inflammation [73].

5 Non-Enzymatic Functions of DPP8 and DPP9

Early investigations using overexpression in the HEK-293T epithelial cell line showed potential roles for DPP8 and DPP9 in apoptosis, wound healing and cell migration. In vitro overexpression of DPP8 and DPP9 resulted in impaired cell adhesion, migration and monolayer wound healing for DPP9 and impaired migration and wound healing for DPP8, suggesting that those outcomes may result from direct protein–protein interactions or via altered expression of other proteins involved in cell adhesion and migration [78]. Both DPP8 and DPP9 also enhance apoptosis. Using enzyme-negative mutants of each protein, the role of DPP8 and DPP9 in apoptosis appeared to be independent of enzymatic activity in HEK-293T cells.

Recent work showed that apoptosis and cell death were unaffected by DPP9 knock-down or enzyme inhibition, but found that enzyme inhibition or gene silencing of DPP9 in Huh7 cells resulted in less cell mobility and adhesion compared to control cells [79], indicating that DPP9 enzymatic function is important for these processes. Differences between that recent study [79] and the early study [78] may reflect that overexpression data can be cell-type specific [30] or can be misleading.

A non-enzymatic activity that needs further exploration is the association of H-Ras with DPP9 [62]. Whether there is direct binding between these two proteins and the functional significance of this association are unknown.

6 Inhibition of DPP8 and DPP9

Many early DPP4 inhibitors were later found to also inhibit DPP8 and DPP9, raising the question of which functions attributed to DPP4 activity should be attributed to DPP8 and/or DPP9. This has led to the development of many inhibitors that are selective for DPP8 and DPP9 over DPP4 and FAP [80–85], but are not selective for DPP8 or DPP9 alone. While some isoindoline inhibitors were originally thought to inhibit DPP8 alone [82, 86], these were later shown to inhibit both DPP8 and DPP9 [81].

The safety of targeting DPP8/9 activity for therapeutic use has been controversial, with toxic effects reported in rats with the use of the DPP8/9 selective inhibitor, UAMC00132 [80], while a rebuttal study on rats and mice using vildagliptin at high doses that inhibit DPP4, DPP8 and DPP9 showed no toxicity or mortality in animals [87]. Moreover, the compound 1G244 does not cause pathological symptoms when inhibiting DPP8 and DPP9 [81]. With the recent design of two analogues of 1G244 that are 10-fold selective for DPP8 over DPP9 [84], there is good progress towards selectively differentiating between these two enzymes by inhibition of either DPP8 or DPP9, rather than both.

Alternative approaches to chemical inhibitors have been examined for targeting DPP4 and FAP. As the DPPs have non-enzymatic functions, antibodies might block cell surface or extracellular protein-binding interactions, or might remove DPP-expressing cells. Immunotargetting DPP4 is an example [88–90]. A separate approach involved a non-substrate non-apeptide, the HIV-1 Tat protein, the first natural inhibitor of DPP4, as an immunosuppressor [91]. Whether non-apeptides or antibodies can enter cells to act on cytoplasmic or nuclear DPP8 and DPP9 is unknown.

More recently, it was found that, while the small ubiquitin-like protein modifier SUMO1 interacts with DPP9 leading to allosteric activation of the peptide [70], the E67-interacting loop peptide acts as a non-competitive inhibitor [71]. This highlights the potential modulation of enzyme activity by peptides that mimic interaction surfaces. Targeting the non-enzymatic activities of DPP8 and DPP9 as a therapeutic approach would require further identification and analyses of protein-binding partners, such as those required for the localisation of DPP9 to the leading edge of moving cells [79].

7 Biological Functions of DPP8 and DPP9

Knowledge of the physiological functions of DPP8 and DPP9 is emerging with investigations in normal homoeostasis and pathophysiological conditions. Data on enzymatic activity, substrate specificity and cell and tissue distribution of these proteases provide some guidance regarding their biological functions in cell survival, cell biology, disease, inflammation and cell-mediated and humoral immunity.

7.1 DPP8 and DPP9 in Immunity

7.1.1 DPP8 and DPP9 in Innate Immunity

The non-specific defence mechanisms against pathogens include physical barriers, such as skin integrity, blood-borne chemicals and certain immune cells. DPP8 and DPP9 have been shown to have extensive in vivo expression in epithelial cells of the gut and skin and normal immunological tissues and also are expressed by all major lymphocyte populations [42, 56, 63]. DPP8 and DPP9 have also been shown to be upregulated upon B or T cell activation [63]. Several T cell leukaemia cell lines highly express DPP8 and DPP9 mRNA transcripts [4].

DPP8/9 enzyme activity has been found in human lymphocytes, monocytes, Jurkat and U937 cells [65] and also in human and mouse primary leucocytes and B and T cell lines [42, 80, 92]. DPP9 is at low levels in both M1 and M2 macrophages derived from U937 cells but abundant in the macrophage-rich regions of plaques [64, 93]. Using the mouse macrophage cell line J774 and bone marrow-derived monocytes and macrophages, an inhibitor of DPP8 and DPP9 has been shown to lessen mouse M1 macrophage activation [64].

Natural killer (NK) cells play a major role in host rejection of both tumours and virally infected cells in the innate immune system. An early study showed that inhibition of DPP4 enzyme activity results in the suppression of stimulatory cytokines causing a reduction of DNA synthesis and cell cycle progression [94]. However, the inhibitors used in that study were later shown to also inhibit DPP8/9 activity [80]; therefore, those effects were probably DPP8/9 mediated. Further support for that conclusion derives from the finding that the presence or absence of DPP4 on the cell surface of NK cells does not influence the natural cytotoxicity of these cells [95]. In a lung metastasis model, NK cytolytic function against tumour cells was less in DPP4-deficient than in WT rats [96].

7.1.2 DPP8 and DPP9 in Adaptive Immunity

The antigen-specific adaptive immune response is complex and includes formation of peripheral and tissue-resident memory leucocytes that improve the efficiency of future responses, and depends upon antigen presentation and recognition. DPP9 is the dominant protease that degrades the antigenic peptide $RU1_{34–42}$ such that downregulation of DPP9 increases the presentation of this antigen [69].

While mouse splenic B lymphocytes express little DPP4 mRNA, DPP8 and DPP9 mRNA are expressed at greater levels and stimulation with pokeweed mitogen and lipopolysaccharide of mouse splenocytes and Jurkat T- and Raji B-cell lines upregulates both proteins [63]. DPP8 and DPP9 mRNA are downregulated by dithiothreitol treatment and upregulated by mitomycin c treatment of Raji cells. Contrary to this, human Jurkat cells or peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin do not change DPP8 or DPP9 expression levels [48].

DPP8 and DPP9 have each been detected by in situ hybridisation in lymphocytes in the mantle and paracortical zones of human lymph node and interfollicular cells of baboon spleen, and DPP8/9 activity has been detected in baboon spleen and

Jurkat cells [56]. Activation and proliferation of immune cells appears to be influenced by DPP8 and DPP9 enzymatic activity, and selective DPP8/9 inhibition can reduce cytokine production due to the induction of TGF-β secretion, as well as DNA synthesis and T cell proliferation [80, 97]. This is suggestive of DPP8/9 enzyme activity having important roles in the regulation of immune function.

7.2 DPP8 and DPP9 in Cell Biology and Cell Survival

Both DPP8 and DPP9 are involved in cell-extracellular matrix interactions, but DPP8 does not influence cell adhesion [78]. While DPP8 and DPP9 are very similar intracellular enzymes and both ubiquitously expressed, DPP9 appears to have a more pronounced role in cell biology.

DPP9 mRNA is elevated in cartilage from osteoarthritis patients [98]. A study of normal and keloid-derived skin fibroblasts in vitro showed that inhibitors of DPP8 and DPP9 could suppress fibroblast proliferation and decrease secretion of both the fibrogenic cytokine TGF-β_1 and procollagen type 1, which is important in the treatment of fibrotic skin disorders and keloid scar [99].

In sarcoma cell lines, NPY-driven tumour cell death mediated by the nuclear protein modifying enzyme poly(ADP-ribose) polymerase (PARP-1) and apoptosis-inducing factor can be abolished by the overexpression of DPP8 and DPP9 and enhanced by downregulating these proteases [77]. In contrast, overexpression of DPP9 in epithelial tumour cell lines is anti-proliferative and enhances intrinsic apoptosis [62, 78]. DPP9 overexpression can also cause less epidermal growth factor (EGF)-mediated Akt pathway activation in HepG2 cells but has not been observed in cells stimulated with other growth factors, suggesting that this activation was growth factor dependent [62]. Since experiments in which DPP9 is overexpressed on the one hand and orgene silenced or inhibited on the other has produced differing data, the pro- or anti-apoptotic activity of this enzyme may depend on its in vitro culture environment and/or the cell type in which the experiment is undertaken.

The DPP inhibitor vildagliptin synergistically enhances parthenolide's action in leukaemia, lymphoma and primary human acute myeloid leukaemia cell lines [100]. This synergy was due to the inhibition of DPP8 and DPP9 rather than DPP4, suggesting that DPP8 or DPP9 inhibition might be a chemosensitising agent for leukaemia cells, as has been found for FAP in fibrosarcoma [101].

The use of a wide variety of cell lines and tissues can provide insights into the physiological and pathological roles of DPP8 and DPP9 in cell behaviour and survival.

7.3 DPP8 and DPP9 in Disease and Inflammation

There is increasing evidence for an association of DPP8 and DPP9 with disease pathogenesis (Fig. 6). In a gene expression profile study of human hepatocellular

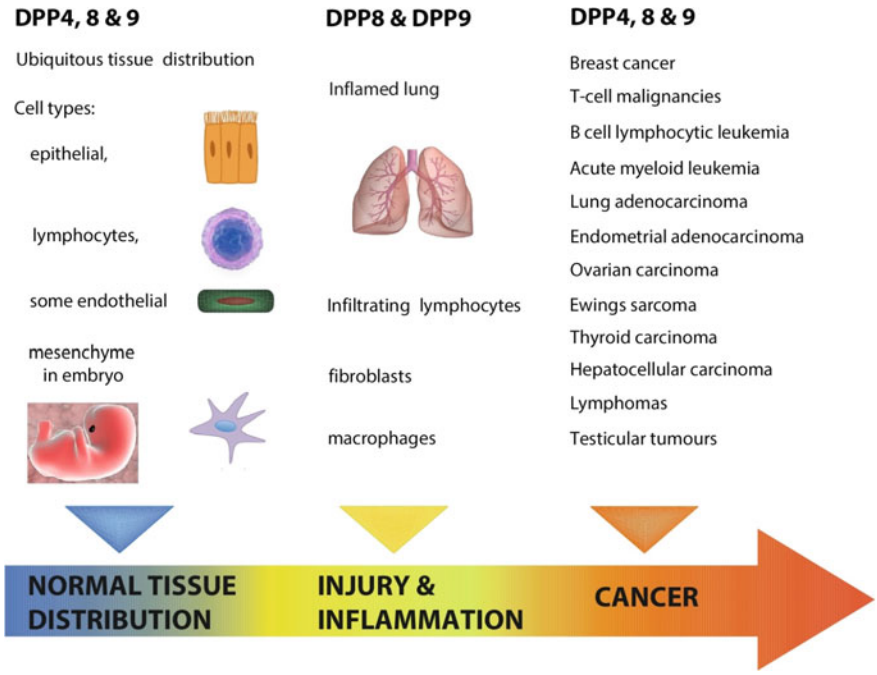

Fig. 6 Summary of studies that have detected mRNA, protein or enzyme activity of DPP4, DPP8 and DPP8 in tissues or cells without disease and increased levels in disease

carcinoma (HCC) tissue, mRNA of DPP9 showed differential expression and upregulation between non-tumour HCC-bearing liver and normal liver [102].

Expression profiling of breast and ovarian carcinoma cell lines, along with 293T and HeLa cell lines, showed ubiquitous but differential expression of DPP8 and DPP9 mRNA and protein across these cell lines. There was poor correlation between mRNA transcript and protein levels for both DPP8 and DPP9, which suggests that DPP8 and DPP9 may be regulated post-transcriptionally in breast and ovarian cancer cell lines [66]. This contrasted with normal mouse tissues, where mRNA and enzyme levels of DPP8 and DPP9 were associated [56].

DPP8 mRNA expression is greater than other DPPs in breast and ovarian cancer cell lines [66], and DPP9 mRNA is abundant in chronic myelogenous leukaemia (K-562) and immortalised cervical cancer (HeLa) cells [43], suggesting that DPP8 and DPP9 may have roles in tumour pathogenesis.

DPP8 and DPP9 mRNA is in tumour infiltrating lymphocytes and DPP9 mRNA is upregulated in human testicular tumours [56]. DPP activity inhibition partially attenuates dextran sulphate sodium (DSS)-induced colitis in mice, and DPP8 mRNA is differentially expressed during colitis development, which is suggestive of role of DPP8 in inflammatory bowel disease [103]. DPP8 mRNA and protein

expression are significantly upregulated in B-cell chronic lymphocytic leukaemia (B-CLL) cells compared to normal tonsil B lymphocytes, which suggests that DPP expression may have biological relevance in B-CLL disease states [36].

Meningiomas are inter-cranial tumours derived from the arachnoid cap cells and include a wide variety of subtypes. DPP8/9 enzymatic activity has been detected in all benign meningiomas, with elevated levels in atypical meningiomas that are associated with greater cell proliferation [61]. An aggressive group of human paediatric malignancies, ESFT, are driven by an aberrant transcription factor that upregulates specific target genes including NPY. ESFT cell death is stimulated by exogenous and endogenous NPY; however, this effect is prevented by cleavage of NPY by cell surface DPP4 and intracellular DPP8 and DPP9. Thus, DPPs are survival factors for EFST and may become potential therapeutic targets for these tumours [77].

A pathophysiologically significant role for DPP8 and DPP9 in asthma is suggested by data from a rat asthma model in which DPP8 and DPP9 and DPP8/9 enzyme activity were primarily detected in the bronchial epithelium of the airways, particularly with allergy-like inflammation [59]. DPP8 and DPP9, along with other DPPs, are elevated in human articular cartilage in osteoarthritis patients, which implicates all of these enzymes in the cascades leading to cartilage breakdown and/or collagenolysis that occurs in arthritis [104].

The expression, localisation and activity patterns of DPP8 and DPP9 have been examined in a model of transient and unilateral cerebral ischaemia in rats [105]. That study found that mRNA expression of DPP9 was diminished in the ischaemic region of the brain at 6 h and 3 days after induced ischaemia, whereas DPP8 levels remained the same at all time points. Also, DPP8 was present in activated microglia and macrophages at day 3 post-ischaemia and in astroglial cells at day 7 post-ischaemia [105]. Those data suggest that DPP8 and DPP9 have potential roles in cerebral inflammation.

As a role for DPP4 in atherosclerosis emerged [106], it was important to investigate the expression and role of DPP8 and DPP9. While DPP4 is only present in endothelial cells of plaque, DPP8 and DPP9 are also in macrophages with a significant upregulation of DPP9 in both pro-inflammatory M1 and anti-inflammatory M2 macrophages during monocyte-to-macrophage differentiation [93]. Primary endothelial cells from aortic, endocardial and cardiac microvascular regions of the rat heart contain DPP8/9 enzymatic activity, with DPP8 protein more abundant than DPP9 [60]. The localisation and expression of DPP8 and DPP9 proteins in endothelia have spatial heterogeneity, with DPP8 more highly expressed in the cardiac microvascular endothelium and DPP9 predominant in the human carotid artery endothelial cells. DPP8 and DPP9 are abundant in the macrophage-rich regions of human and mouse atherosclerotic plaques [64, 93]. M1 macrophages have a role in atherogenesis, so those data suggest potential therapeutic prospects in atherosclerosis and/or plaque rupture by inhibition of DPP9.

A genome-wide association study that focused on human pulmonary fibrosis identified *DPP9* as one of several novel susceptibility loci. Although the evidence for greater expression of *DPP9* in lung tissue of pulmonary fibrosis cases compared

to controls was slight, this group speculated that DPP9 may be involved in the integrity of lung epithelia via cell-to-cell adhesion [107]. This finding needs further investigation of whether DPP9 has a role in the development of pulmonary fibrosis.

8 DPP4 Family Mouse Phenotypes

8.1 Mouse Models

As the mouse genome is amenable to genetic manipulation, many studies involve the use of gene knockout (GKO) mice as models for investigation of the biological properties of proteins in a physiological and/or pathophysiological context. In GKO mice, the protein of interest is absent from the resultant mouse. In the DPP4 gene family, GKO mice have been created for both DPP4 and FAP. The DPP4-GKO mouse was produced by a targeted inactivation in the *DPP4* gene and resulted in healthy mice [108]. Similarly, the FAP-GKO mouse was constructed through exon deletion and also created fertile mice with no overt developmental or haematological defects or increased cancer susceptibility [109]. It has been speculated that, in the absence of these proteins, other proteins are upregulated and have compensatory roles. To date, no DPP8-GKO mice have been reported.

8.1.1 DPP9 Gene Knock-in (GKI) Mouse

Unlike GKO mice where the entire protein is absent from the mouse, in a GKI mouse the protein of interest is present but inactivated; thus, it is still able to fulfil all functions apart from the one that has been removed. By targeting the DPP9 protein catalytic site with a serine to alanine point mutation, we were able to create a DPP9 gene knock-in mouse (DPP9-GKI) that lacks DPP9 enzymatic activity while maintaining normal DPP9 protein structure and thus the non-enzymatic functions of DPP9 [52]. This DPP9-GKI mouse exhibits neonate lethality, suggesting that DPP9 enzymatic activity is essential for early neonatal survival in mice [52]. However, no morphological defect or cause of death has been identified. Further studies using neonatal liver and gut from DPP9-GKI mice compared to WT have shown that DPP9 enzymatic activity influences the expression of many genes [110].

Taqman PCR arrays and qPCR analyses of neonatal liver revealed differential expression of genes involved in cell growth (epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), vascular endothelial growth factor-A (VEGF-A), innate immunity (tumour necrosis factor (TNF)-α, interleukin-1 β, IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha)) and metabolism signalling pathways, such as insulin receptor substrate-2 (INS-2). Neonatal liver and gut showed differential expression of the master transcription coactivator peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) and genes involved in long-chain-fatty-acid uptake and esterification, long-chain fatty acyl-CoA binding, trafficking and transport into

mitochondria, lipoprotein metabolism, adipokine transport and gluconeogenesis in the DPP9-GKIhomozygote neonatal mice compared to WT. In addition, immunoblots showed that DPP9 enzyme activity can modulate AMP-activated protein kinase phosphorylation in the Huh7 human hepatoma cell line [110].

The differential expression of genes involved in cell growth, innate immunity, lipid metabolism and gluconeogenesis suggests roles for DPP9 enzymatic activity in the regulation of immune and metabolic pathways in the neonate [110].

9 Conclusion

DPP9 is emerging as an interesting and unique atypical serine protease that has potential roles in tumours, leukaemia, atherosclerosis, inflammation and energy metabolism and in T, B and NK cells and endothelial cells. Numerous studies have been reported now that detail DPP9 expression and its upregulation in brain, joint, liver, lung and gut inflammation. However, a dominant homoeostatic or disease role for DPP9 has not been identified. Despite strong expression in muscle, no function in muscle or heart has arisen. The enzyme activity of DPP9 has been implicated in neonate survival, metabolism and immune system, and in macrophage function, EGF receptor signalling and cell migration.

The impediments to a greater understanding of DPP9 are the lack of any selective enzyme inhibitor, specific enzyme assay or adult GKO or GKI mice.

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