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Prions

Prion Protein Endoproteolysis: Cleavage Sites, Mechanisms and Connections to Prion Disease

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ABSTRACT

Highly abundant in neurons, the cellular prion protein (PrP$^{\text{C}}$) is an obligatory precursor to the disease-associated misfolded isoform denoted PrP$^{\text{Sc}}$ that accumulates in the rare neurodegenerative disorders referred to either as transmissible spongiform encephalopathies (TSEs) or as prion diseases. The ability of PrP$^{\text{C}}$ to serve as a substrate for this template-mediated conversion process depends on several criteria but importantly includes the presence or absence of certain endoproteolytic events performed at the cell surface or in acidic endolysosomal compartments. The major endoproteolytic events affecting PrP$^{\text{C}}$ are referred to as α- and β-cleavages, and in this review we outline the sites within PrP$^{\text{C}}$ at which the cleavages occur, the mechanisms potentially responsible and their relevance to pathology. Although the association of α-cleavage with neuroprotection is well-supported, we identify open questions regarding the importance of β-cleavage in TSEs and suggest experimental approaches that could provide clarification. We also combine findings from in vitro cleavage assays and mass spectrometry-based studies of prion protein fragments in the brain to present an updated view in which α- and β-cleavages may represent two distinct clusters of proteolytic events that occur at multiple neighbouring sites rather than at single positions. Furthermore, we highlight the candidate proteolytic mechanisms best supported by the literature; currently, despite several proteases identified as capable of processing PrP$^{\text{C}}$ in vitro, in cell-based models and in some cases, in vivo, none have been shown conclusively to cleave PrP$^{\text{C}}$ in the brain. Addressing this knowledge gap will be crucial for developing therapeutic interventions to drive PrP$^{\text{C}}$ endoproteolysis in a neuroprotective direction. Finally, we end this review by briefly addressing other cleavage events, specifically ectodomain shedding, γ-cleavage, the generation of atypical pathological fragments in the familial prion disorder Gerstmann–Sträussler–Scheinker syndrome and the possibility of an additional form of endoproteolysis close to the PrP$^{\text{C}}$ N-terminus.

1 | Introduction

Misfolding of the cellular prion protein (PrP$^{\text{C}}$) into protease-resistant conformations (collectively referred to here as PrP$^{\text{Sc}}$) followed by template-mediated conversion of additional PrP$^{\text{C}}$ molecules is integral to the pathogenesis of the rare but fatal neurodegenerative disorders known either as transmissible spongiform encephalopathies (TSEs) or prion diseases (McKinley, Bolton, and Prusiner 1983; Prusiner 1982). PrP$^{\text{C}}$ is first synthesized as a pro-protein with a cleavable N-terminal

Abbreviations: AD, Alzheimer's disease; ADAM, A disintegrin and metalloproteinase; Aβ, amyloid-beta; CJD, Creutzfeldt–Jakob disease; DPP4, dipeptidyl peptidase-4; ER, endoplasmic reticulum; FAP, fibroblast activation protein; FL, full-length; GPI, glycophosphatidylinositol; KO, knockout; OR, octarepeat; PK, proteinase K; PrP$^{\text{C}}$, cellular prion protein; PrP$^{\text{Sc}}$, misfolded prion protein; recPrP, recombinant PrP; ROS, reactive oxygen species; SMB (cells), scrapie-infected mouse neuroblastoma (cells); TSEs, transmissible spongiform encephalopathies; WT, wild-type.

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signal peptide (Basler et al. 1986) for targeting the endoplasmic reticulum (ER). After translocation into the ER, a C-terminal signal peptide is removed, enabling the addition of a glycophosphatidylinositol (GPI) anchor to the mature PrP^C, which consists of ~208 amino acids (residues 23–230; human numbering used here and throughout the introduction). N-linked glycans are also usually added at Asn181 and Asn197. After exiting the ER, PrP^C progresses into the Golgi apparatus, where the glycans undergo maturation (Caughey et al. 1989) and the protein is sorted for secretion to the cell surface. Once there, PrP^C attaches to the extracellular side of the cell membrane via its GPI anchor (Stahl et al. 1987). PrP^C is most abundant on the surface of neurons but is also expressed by various cell types both inside and outside of the nervous system (Castle and Gill 2017).

The PrP^C N-terminal domain is flexibly unstructured, but can acquire stable structure, most notably upon binding to metal ions such as Cu^{2+} (Chattopadhyay et al. 2005). Although the PrP^C sequence as a whole is well-conserved among mammalian species, this is particularly true of the N-terminal domain (Kim, Lee, and Lee 2008), which begins with a short sequence of mostly positively charged residues (KKRPK). Two hexarepeats (GGS/NRYP) follow shortly afterwards (residues 34–39 and 45–50), with hexarepeat 2 immediately preceding the octapeptide repeat (OR) domain of residues 51–91 (4 × PHGGGWGQ preceded by an additional imperfect repeat) (Bazan et al. 1987). Connected to the N-terminal domain via a hydrophobic linker (residues ~112–133), the globular C-terminal domain contains three α-helices, an antiparallel two-stranded β-sheet and interconnecting loops, with a solvent-protected disulphide bond between Cys179 and Cys214 (Haire et al. 2004; Riek et al. 1996). These structural features of PrP^C are visualized in Figure 1. The pathogenic misfolding process greatly increases β-sheet-content (Pan et al. 1993), leading to residues ~90–230 acquiring protease resistance (Prusiner et al. 1984).

Knowledge of the PrP^C sequence and its structure is useful when considering the various endoproteolytic cleavages that the protein can undergo. The most well-studied fragmentation events are referred to as 'α' and 'β'. α-Cleavage separates the N-terminal domain from the C-terminal domain and hydrophobic linker, whereas β-cleavage occurs closer to the N-terminus, resulting in a longer C-terminal fragment. Although we will end this review by briefly covering other reported cleavage events, our focus is on the α- and β-cleavages as they may represent a molecular switch in TSE pathogenesis, with α-cleavage widely regarded as anti-pathogenic and β-cleavage as potentially pro-pathogenic.

2 | Alpha-Cleavage

2.1 | Cleavage Sites Within PrP^C

The C-terminal fragment of α-cleavage, C1, was initially discovered and named as part of an investigation into the forms of PrP present in post-mortem human brain tissue (Chen et al. 1995). N-terminal radiosequencing data from the same study suggested that α-cleavage occurs at Lys110↓His111 or His111↓Met112 (note that Val tends to be substituted for Met at the equivalent position in other mammalian species). Later, α-cleavage was shown to be highly resistant to amino acid changes in the vicinity of these cleavage sites (Oliveira-Martins et al. 2010). Specifically, mutating both Lys109 and His110 of mouse PrP^C (equivalent to Lys110 and His111 in human PrP^C) to either Ala or Asp residues had minimal impacts on C1 fragment levels in cultured cells (Oliveira-Martins et al. 2010). Experiments with additional deletion mutants in the same study showed that the space occupied by residues 105–120 is crucial, but the individual amino acid identities are surprisingly unimportant. For example, deleting the entire 105–120 region largely eliminated C1 production, while deletions of either residues 105–110 or the palindromic sequence AGAAAAGA (112–119) reduced C1 levels by ~50%. In contrast, converting the 112–119 sequence entirely to Gly residues left α-cleavage seemingly unaffected. Furthermore, neither changing the hydrophobicity nor the charge of the 100–129 region had major effects on C1 levels (Oliveira-Martins et al. 2010).

To obtain a comprehensive understanding of PrP^C cleavage sites in vivo, a recent study employed in-gel acetylation of the N-termini of PrP^C fragments immunoprecipitated from mouse brain, followed by in-gel trypsinization and mass spectrometric analysis. High-confidence α-cleavage sites for wild-type (WT) mouse PrP^C were identified at His110↓Val111, Gly113↓Ala114, Ala116↓Ala117 and Ala119↓Val120, as well as additional lower-confidence sites within the region of residues 105–120 (Gomez-Cardona et al. 2023). Interestingly, the previously reported Lys109↓His110 cleavage (mouse PrP numbering scheme) was not detected. Considering these data as a whole, it seems almost certain that more than one protease performs α-cleavage of PrP^C, which is probably why it has proven difficult to identify

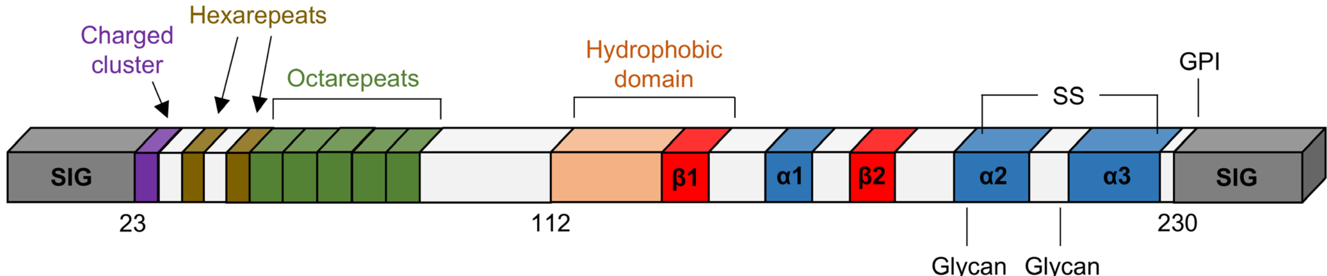

FIGURE 1 | Schematic of PrP^C structure. Key structural features of PrP^C are highlighted, including the charged cluster, hexarepeats and tandem octarepeats of the N-terminal domain, the hydrophobic linker and the C-terminal domain with its three α-helices and two β-strands. A disulphide bond (SS) stabilizes the fold of the globular C-terminal domain. The signal peptides (SIG) are not present in the mature protein that is expressed on the cell surface.

the responsible protease(s) with high certainty. In the next section, we will cover the proteases putatively linked to α-cleavage so far (for brevity, referred to as α-PrPases).

2.2 | Candidate α-PrPases

2.2.1 | ADAM10 and ADAM17

The most well-studied candidate α-PrPases are members of a disintegrin and metalloproteinase (ADAM) family. Initial cell culture experiments showed that the metalloprotease inhibitors *o*-phenanthroline, TAPI and BB3103 reduced (but did not eliminate) N1 secretion (Vincent et al. 2001). In the same study, overexpression of ADAM10 or ADAM17 (also known as tumour necrosis factor-α converting enzyme, TACE) increased constitutive or phorbol ester-regulated N1 secretion, respectively. Converse effects were also observed when ADAM10 or ADAM17 were knocked out in cultured mouse embryonic fibroblasts. Later studies demonstrated that ADAM10 and C1 fragment levels were positively correlated in post-mortem human brain tissue (Laffont-Proust et al. 2005), while inhibition of a signalling pathway involving RhoA-associated coiled-coil containing kinases and pyruvate dehydrogenase kinase isozyme 1 in prion-infected neuronally differentiated 1C11 cells or primary neuronal cultures increased both ADAM17 cell-surface localization and C1 fragment levels (Alleaume-Butaux et al. 2015; Pietri et al. 2013). However, none of these studies conclusively demonstrated that ADAM10 and/or ADAM17 cleave PrP^C directly, while others have found that neither treating with various metalloprotease inhibitors (marimastat, *o*-phenanthroline or TAPI) nor manipulating the levels of ADAM10 or ADAM17 (or ADAM9) in cultured cells by overexpression or siRNA treatment had any effects on C1 fragment levels (Béland et al. 2012; Taylor et al. 2009; Wik et al. 2012). Furthermore, ADAM10 knockout (KO) in mice did not reduce C1 fragment levels in total brain homogenates or primary neuronal cultures (Altmeppen et al. 2011). In fact, given that ADAM10 has been conclusively linked to ectodomain shedding of PrP^C (Altmeppen et al. 2011; Taylor et al. 2009), the originally observed effects of metalloprotease inhibitors on N1 secretion could be explained by changes to shedding, as has been suggested previously (Wik et al. 2012).

In spite of the above controversy, while experiments in which mouse recombinant PrP (recPrP) was incubated with recombinant ADAM10 or ADAM17 failed to show proteolysis at either of the originally identified α-cleavage sites (Lys109↓His110 or His110↓Met/Val111) (McDonald et al. 2014), the same in vitro study found that ADAM10 and ADAM17 could cleave mouse recombinant PrP at Ala119↓Val120; this matches a high-confidence cleavage site identified in a recent mass spectrometry-based analysis of mouse-brain PrP^C referred to previously (Gomez-Cardona et al. 2023). Although cleavage site specificities for ADAMs are somewhat ambiguous, Ala seems to be the preferred residue in the 'P1' position (immediately N-terminal of the hydrolysed peptide bond) for both ADAM10 and ADAM17, while Val is the most highly favoured on the C-terminal side of the cleaved bond ('P1'') for ADAM17 (although not ADAM10) (Caescu, Jeschke, and Turk 2009). Indeed, the cleavage of tumour necrosis factor-α, from which ADAM17 derives its alternative name, occurs at an Ala-Val bond. Therefore, taking the published data on ADAM10 and ADAM17 together, we conclude that although other proteases must also be involved in PrP^C α-cleavage, ADAM17 (and perhaps ADAM10) may contribute to a fraction of C1 production via cleavage at Ala119↓Val120.

2.2.2 | ADAM8

Another ADAM protease linked to PrP^C α-cleavage is ADAM8, but in this case the connection is supported by in vivo data. Specifically, experiments with various transgenic mouse lines expressing different levels of PrP^C, including a line with doxycycline-inducible PrP^C overexpression, showed that ADAM8 protein levels were positively correlated with the ratio of C1 to full-length (FL) PrP^C in skeletal muscle tissue (quadriceps), while ADAM8-null skeletal muscle displayed a dramatic reduction in the C1/FL ratio ($>80\%$) compared with WT control tissue (Liang et al. 2012). In the same study, ADAM8 levels and α-cleavage rates were also positively correlated in the C2C12 myoblast cell line, and an enzymatically active fragment of recombinant human ADAM8 was shown to cleave human recPrP directly, generating a C1-like fragment of the expected size. Direct in vitro cleavage by ADAM8 was later confirmed independently to take place at Lys109↓His110 of recMoPrP, with a minor cleavage site at Ala116↓Ala117 (McDonald et al. 2014). Furthermore, PrP^C is known to bind Cu^{2+} in vivo (Brown et al. 1997), and the presence of bound copper ions (≥ 2 M equivalents) switched the relative efficiencies of cleavage by ADAM8 at the two aforementioned sites, presumably due to structural changes caused by differing copper coordination arrangements (McDonald et al. 2014).

Nonetheless, the brain rather than skeletal muscle is most relevant in the context of TSEs, and while ADAM8 is expressed in the brain (Kelly et al. 2005), results on the effect of ADAM8 KO on C1 levels in the mouse brain have not been published. A review article by two authors of the original study linking ADAM8 to α-cleavage does, however, refer to unpublished data indicating 'a less prominent role for ADAM8 in the α-cleavage of PrP^C in the brain' (compared with skeletal muscle) (Liang and Kong 2012). Therefore, the contribution of ADAM8 towards C1 production in the brain remains unclear.

2.2.3 | Plasmin

Another protease shown to perform α-cleavage of recPrP is plasmin (Kornblatt et al. 2003; Praus et al. 2003), a trypsin-like serine protease that derives from the pro-protein plasminogen. In fact, PrP^C may itself promote conversion of plasminogen to plasmin by tissue-type plasminogen activator (Praus et al. 2003). Given that trypsin-like proteases prefer Lys or Arg residues in P1, with plasmin seeming to favour Lys in particular (Harris et al. 2000), it is highly plausible that plasmin could cleave PrP^C at Lys109/110↓His110/111, as was the case in vitro (Kornblatt et al. 2003; Praus et al. 2003). A recent study confirmed the in vitro α-PrPase activity of plasmin but also showed that adding recombinant human plasmin to N2a neuroblastoma cells dramatically increased C1 fragment levels (Mays et al. 2022). However, even though plasminogen-null mice may have slightly lower C1 levels in kidney and lung tissues, C1 fragment levels

in the brain were completely unaltered by plasminogen KO (Barnewitz et al. 2006), which suggests either that plasmin does not cleave PrP^C in the brain or that other proteases compensate for its absence.

In our recent publication, a cell-based screen of 130 protease inhibitors identified camostat mesylate as the only compound that specifically reduced C1 levels without affecting levels of the β-cleavage fragment C2 (Castle et al. 2023). Given that camostat mesylate is a broad-spectrum inhibitor of trypsin/chymotrypsin-like serine proteases that are known to inhibit plasmin (Iwata et al. 2023), our finding indirectly supports the role of plasmin in PrP^C α-cleavage, although another serine protease could also be involved. Interestingly, C1 levels were not affected by nafamostat mesylate, which ought to inhibit plasmin at a 20 μM concentration (Al-Horani and Desai 2014), as used in our study (Castle et al. 2023).

2.2.4 | Calpains

The final candidates for α-PrPases are the calpain family. As far as we are aware, evidence for their involvement in α-cleavage derives from a single study that used cells expressing PrP^C with N-terminal DsRed and C-terminal GFP fusions, respectively. Through imaging and immunoblotting analyses, the authors showed that treatment with the endogenous calpain inhibitor calpastatin reduced PrP^C α-cleavage, while direct cleavage of recPrP in vitro by recombinant calpain 1 was also demonstrated (Hachiya et al. 2011). However, it is surprising that leupeptin did not show a similar effect to calpastatin in the cell model given that the ~23 μM concentration used should inhibit calpain activity completely (Saito and Nixon 1993). In addition, while α-cleavage of endogenous PrP^C in the same cells was also clearly inhibited by calpastatin, the physiological relevance of these findings is debatable given that both the DsRed–PrP^C–GFP fusion protein and the endogenous PrP^C appeared to be unusually concentrated in the Golgi apparatus, perhaps because of stress induced by overexpressing a form of PrP^C three times its normal size (due to the DsRed and GFP moieties in the fusion proteins). Furthermore, a later study using a traumatic brain injury mouse model showed that treatment with a calpain inhibitor did not affect PrP^C fragmentation (Rubenstein et al. 2018), while the same was true for several calpain inhibitors tested in the aforementioned protease inhibitor screen, including MDL 28170, calpain inhibitor II (ALLM) and PD 151746 (Castle et al. 2023).

2.2.5 | Summary of the Candidate α-PrPases

None of the candidate α-PrPases are proven conclusively to cleave PrP^C in the brain, which, with regard to TSEs, is the primary tissue of interest. Recent studies do allow us to conclude the following, however: (1) rather than occurring at a single site, what we refer to as α-cleavage is most likely a collection of cleavage events occurring within the 109–121 region of PrP^C and (2) by taking into account the cleavage site specificities of the candidate α-PrPases as well as in vitro experiments with recombinant proteins, ADAM8 and plasmin can be plausibly linked to cleavage at Lys109↓His110 (mouse PrP^C numbering), ADAM8 to cleavage at Ala116/Ala117 and ADAM10 (and possibly ADAM17) to cleavage at Ala119↓Ala120 (Figure 2). This knowledge provides an opportunity to overcome the difficulties caused by the apparent redundancy in α-PrPase activity, that is, the relative contributions of different proteases to PrP^C α-cleavage in the brain could be elucidated by further mass spectrometry-based analysis of PrP^C fragments immunoprecipitated from brain tissue (or human neurons derived from induced pluripotent stem cells) in which the candidate α-PrPases have been individually ablated. The disappearance (or reduction in frequency) of an α-cleavage site present in controls would provide strong evidence for α-PrPase activity of that particular protease in the brain. Of course, the putative α-PrPases currently identified may not account for the full spectrum of α-cleavage activity and further candidates may need to be sought. Site heterogeneity might also derive from a single endoproteolytic event followed by aminopeptidase trimming, thereby generating a range of C-terminal fragments with slightly different sizes.

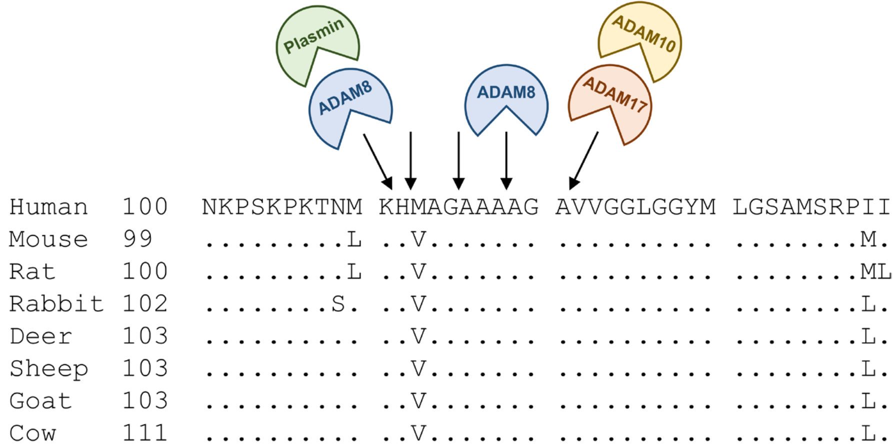

FIGURE 2 | α-Cleavage sites and putative α-PrPases. Alignment of PrP^C sequences from various mammalian species (using NCBI blastp) showing that the region containing the α-cleavage sites is well-conserved at the amino acid level, with dots used to denote sequence identity. Arrows indicate the cleavage sites identified by N-terminal radiosequencing of fragments purified from post-mortem human brain tissue (Chen et al. 1995) or by mass spectrometry after immunoprecipitation from mouse brain (Gomez-Cardona et al. 2023). Potentially responsible α-PrPases are indicated based on data from in vitro cleavage assays (McDonald et al. 2014; Praus et al. 2003).

2.3 | Subcellular Localization of α-Cleavage

As with many features of α-cleavage, its subcellular localization remains controversial. Initial analysis of chicken PrP^C expressed in N2a neuroblastoma cells suggested that α-cleavage occurs after recycling from the cell surface into an acidic endosomal compartment (Shyng, Huber, and Harris 1993), although the authors do not completely rule out cell-surface cleavage followed by internalization of C1. A slightly later study supports the cell-surface hypothesis, with α-cleavage occurring in cholesterol-rich membrane microdomains (Taraboulos et al. 1995). However, α-cleavage has also been localized to the secretory pathway (Tveit et al. 2005), with temperature-block experiments from one study specifically pointing towards the Golgi (Walmsley et al. 2009), whereas similar experiments in a separate study suggest cleavage takes place *after* export from the Golgi (Zhao et al. 2006).

If we consider the identities of the candidate α-PrPases, then cleavage at the cell surface seems most likely. For example, ADAMs are transmembrane proteins thought to be inactive during their passage through the early secretory pathway (Wong et al. 2015) and are typically involved in ectodomain shedding from cell-surface proteins or cleavage of extracellular matrix proteins (Lambrecht, Vanderkerken, and Hammad 2018). Meanwhile, plasminogen is present in the extracellular space but binds to the surface of astrocytes (Briens et al. 2017) and neurons (Ho-Tin-Noé et al. 2009), where it can be converted to active plasmin.

Irrespective of whether α-cleavage takes place at the cell surface or not, C1 is abundant on the outer side of the cell membrane, to which it is attached via a GPI anchor (Chen et al. 1995; Harris et al. 1993; Laffont-Proust et al. 2006) in the same manner as FL PrP^C (Stahl et al. 1987). In contrast, the N-terminal fragment N1 lacks the GPI anchor for retention at the plasma membrane (Harris et al. 1993). Here, the possibilities are that N1 exists in a diffusible form in the extracellular space/brain parenchyma, or can remain cell-associated by engagement with cell-surface proteins to perform physiological functions, which may include myelin maintenance (Kuffer et al. 2016) and modulation of other cell signalling processes (Carroll et al. 2020; Haigh et al. 2009b).

2.4 | Relevance to the Pathogenesis of Neurodegenerative Diseases

PrP^C α-cleavage is of interest mainly because neither fragment generated contains the entire proteinase K (PK)-resistant domain of PrP^{Sc}, which corresponds to residues ~90–231 (Prusiner et al. 1984). Furthermore, α-cleavage is a common processing event, with the C1 fragment being highly expressed in the brain – typically at least to the same level as FL PrP^C across multiple animal species (Madsen-Bouterse et al. 2021; Vanni et al. 2023), although C1 may be slightly lower in abundance in the human brain (Laffont-Proust et al. 2005).

Many studies into whether products of PrP^C fragmentation undergo disease-associated structural conversion in a similar manner to FL PrP^C have focused on C-terminal fragments, which contain some of the residues represented in PK-resistant PrP^{Sc} fragments. In the case of α-cleavage, consistent data have accumulated indicating that C1 has a greatly diminished ability to misfold. For example, seeded conversion of N-terminally truncated recPrP approximating C1 is considerably less efficient than that of FL recPrP (Lawson et al. 2001), while C1-like recPrP also resists the spontaneous refolding into a highly heat-stable, β-strand-rich structure that occurs to FL recPrP when its disulphide bond is reduced by exposure to low-pH acetate buffer (Johanssen et al. 2014). Similarly, cell lines with higher levels of C1 relative to FL PrP^C were more resistant to infection with the mouse-adapted human prion strain M1000 (Lewis et al. 2009), while expression of mouse PrP^C with the D177N substitution (equivalent to D178N in humans), which causes the inherited TSE fatal familial insomnia, was associated with dramatically lower levels of C1 production (~70% reduction) compared with WT PrP^C (Oliveira-Martins et al. 2010). Moving onto in vivo studies, mice expressing $PrP^{\Delta 23-111}$ (approximating constitutive C1 expression) on a *Prnp*-null background did not succumb to the disease within a year of intracerebral inoculation with the mouse-adapted scrapie prion strain RML even though WT mice reached disease endpoint around 172 days post-infection (Westergard, Turnbaugh, and Harris 2011a). In fact, experiments in mice expressing $PrP^{\Delta 23-111}$ on a WT background showed that C1 acts as a dominant-negative inhibitor of TSE pathogenesis (although, interestingly, not infectivity), leading to extended incubation periods compared to mice solely expressing endogenous PrP^C (Westergard, Turnbaugh, and Harris 2011a).

Notwithstanding the findings detailed above, we note that PK-resistant, C1-like fragments have been observed on rare occasions. For example, a serendipitous finding from investigations into the artificially engineered PrP^C mutant known as 'S3' is that interaction between S3 PrP^C and cobalamin can trigger spontaneous formation of detergent-insoluble, PK-resistant PrP, including a form with similar electrophoretic mobility to C1 (Daude et al. 2022); S3 PrP^C has a compact N-terminal conformation, which was designed to restrict copper binding to a specific geometry as opposed to the more flexible copper coordination of WT PrP^C or metal-free WT PrP^C apoprotein (Lau et al. 2015). A C1-like fragment with spontaneously acquired PK resistance was also observed when ovine PrP^C lacking residues 190–196 was expressed in the RK13 cell line (Munoz-Montesino et al. 2020). However, these are atypical prions produced from mutated forms of PrP^C not known to exist in nature. As far as we are aware, the only report of C1-like, PK-resistant PrP^{Sc} in vivo comes from a study of two cows tested as part of a bovine spongiform encephalopathy surveillance program (Serra et al. 2017). These cows never displayed clinical signs of the disease during their lifetimes, and it is not clear whether pathogenic conversion of physiologically generated C1 had taken place or, instead, whether these animals had an atypical prion strain with a smaller protease-resistant domain than normal (i.e., the fragment was generated *after* PK treatment in vitro).

Overall, the consensus in the literature is that upregulation of α-cleavage may attenuate the progression of prion infections, and initial steps in testing this approach have recently been

made. For example, adding the suspected α-PrPase plasmin to persistently prion-infected N2a (ScN2a) cells increased C1 levels dramatically and inhibited formation of PK-resistant PrP^{Sc}, although a high concentration of exogenous plasmin (500 nM) was needed to reduce total PrP^{Sc} levels; slightly lower concentrations were effective against PrP^{Sc} formation from newly synthesized PrP^{C} generated after transfection (Mays et al. 2022). The authors postulate that the requirement for high levels of plasmin may have been due to alpha2-antiplasmin (an endogenous plasmin inhibitor) present in the foetal bovine serum used to supplement the culture medium. Another study showed that peptide aptamers binding to the PrP^{C} central region promote α-cleavage in a variety of cell lines (Corda et al. 2018). These peptide aptamers also display anti-prion properties, which likely derive at least in part from their effects on α-cleavage, although this was not demonstrated explicitly. After these promising initial results, further studies are warranted to establish whether enhancing PrP^{C} α-cleavage through similar or novel mechanisms is protective against TSE pathogenesis in vivo.

In addition to the key role that its misfolding and aggregation plays in TSEs, it is well-established that PrP^{C} is a cell-surface receptor for Alzheimer's disease (AD)-associated amyloid-beta (Aβ) oligomers and may mediate their cellular toxicity through hyper-activation of Fyn kinase (Corbett et al. 2020; Gunther et al. 2019; Jarosz-Griffiths et al. 2019). Indeed, one study comparing the relative importance of different reported receptors for Aβ oligomers found that PrP^{C} was the highest-affinity receptor tested (Smith et al. 2019). Therefore, lowering PrP^{C} levels would likely reduce Aβ oligomer toxicity, and some strategies to reduce total PrP^{C} levels either with small molecules (Mehrabian et al. 2022), antisense oligonucleotides designed against *PRNP* mRNA (Minikel et al. 2020) or an adeno-associated virus-encoded epigenetic editing system (Neumann et al. 2024) have already been developed. An alternative approach is to promote PrP^{C} α-cleavage, given that the reported binding sites of Aβ oligomers are absent in C1 (Chen, Yadav, and Surewicz 2010; Laurén et al. 2009) and that recombinant N1 was shown to protect primary cultures of mouse cortical neurons from toxicity caused by Aβ oligomers (Guillot-Sestier et al. 2012), probably by interacting with the oligomers in the extracellular space and preventing their binding to FL PrP^{C} on the cell surface. This strategy was attempted by a recent study that used one of the peptide aptamers mentioned above that is able to enhance α-cleavage (Ali et al. 2023). Intraventricular infusion of the aptamer into 5xFAD transgenic mice improved memory performance, reduced gliosis and lowered levels of stress kinase activation in the brain. Whether these effects were due to the aptamer enhancing α-cleavage or blocking the major Aβ oligomer binding site on FL PrP^{C} (or both) remains to be established. Nonetheless, the promising initial results from promoting PrP^{C} α-cleavage in TSE and AD models provide an incentive to clarify the proteases responsible for this process, as this knowledge will aid in the development of more targeted therapeutic approaches. Although it is easier to design small-molecule protease inhibitors than activators, protease-activating compounds do exist (Wolan et al. 2009). Furthermore, recent developments in RNA-targeted therapeutics offer an alternative approach (Khorkova et al. 2023), which may allow for controlled upregulation of a suitable PrP^{C}-cleaving protease.

3 | Beta-Cleavage

3.1 | Cleavage Site(s) Within PrP^{C}

Before α-cleavage was first characterized, in vivo truncation of PrP^{Sc} was detected in material purified from the brains of mice infected with the ME7 mouse-adapted scrapie strain, with Edman sequencing of the C-terminal fragments identifying cleavage sites within the OR domain (Hope et al. 1988). The OR domain of mouse PrP is virtually identical to the human form, with only a couple of changes to OR1 in addition to G-to-S substitutions in OR3/4 (Figure 3A). The major C-terminal fragments described in the aforementioned study began with SWG, indicative of HGG↓SWG cleavages within OR3/4, while analysis of less abundant fragments suggested that cleavages also took place at similar positions within OR2/5 (HGG↓GWG or GGG↓WGQ). Further studies confirmed the presence of N-terminally truncated PrP^{Sc} in scrapie-infected mouse neuroblastoma (SMB) cells (Caughey et al. 1991; Taraboulos et al. 1992) and post-mortem human brain tissue from individuals with sporadic Creutzfeldt–Jakob disease (CJD) (Chen et al. 1995). The latter study also introduced the C2 nomenclature to describe N-terminally truncated PrP^{Sc}, in contrast with the shorter C-terminal fragment in normal brain tissue, which the authors named C1. In slight disagreement with the Edman sequencing data, most studies have reported that N-terminal truncation of PrP^{Sc} occurs up to approximately the end of the OR domain (~residue 90), although this is largely based on low-resolution epitope mapping approaches as well as the similarity in electrophoretic mobility (after deglycosylation) to PrP^{Sc} species generated by post-lysis treatment with PK (Chen et al. 1995; Jimenez-Huete et al. 1998; Owen et al. 2007b; Yadavalli et al. 2004). The size of the truncated PrP^{Sc} may also vary slightly between different TSEs (Jimenez-Huete et al. 1998).

Due to the initial association of C2 production with prion infection, the field has tended to regard the appearance of the fragment as restricted to pathological situations. However, although considerably more prominent in TSE cases, the previously referenced studies of post-mortem human brain tissue did detect faint bands of approximately the same size as N-terminally truncated PrP^{Sc} in control brains (Chen et al. 1995; Jimenez-Huete et al. 1998). Moreover, prominent C2 bands of at least equal intensity to FL PrP^{C} have been detected under non-pathological conditions in various mouse or human tissue types, including tonsil, kidney, lung, spleen, pancreas, adipose, muscle and the eye (Ashok et al. 2019, 2018; Ashok and Singh 2018; Asthana et al. 2017; Boufroura et al. 2021; Castle et al. 2023; Jimenez-Huete et al. 1998). To acknowledge this distinction, we will refer hereafter to N-terminally truncated PrP^{Sc} as $C2^{Sc}$ and the physiological cleavage product as C2.

The physiological generation of C2 was named 'β'-cleavage by Mangé et al. (2004). As part of this study, the authors used immunoprecipitation followed by SELDI-TOF mass spectrometry to map β-cleavage of mouse PrP^{C} in cultured cells to Asn96↓Gln97. In contrast, a more recent study found that the majority of β-cleavage in bank vole brain tissue occurs on the N-terminal side of the WGQGG epitope of the antibody 12B2 (which straddles the end of the OR domain) (Vanni et al. 2023). Mass spectrometry-based analysis of PrP^{C} fragments immunoprecipitated from normal mouse

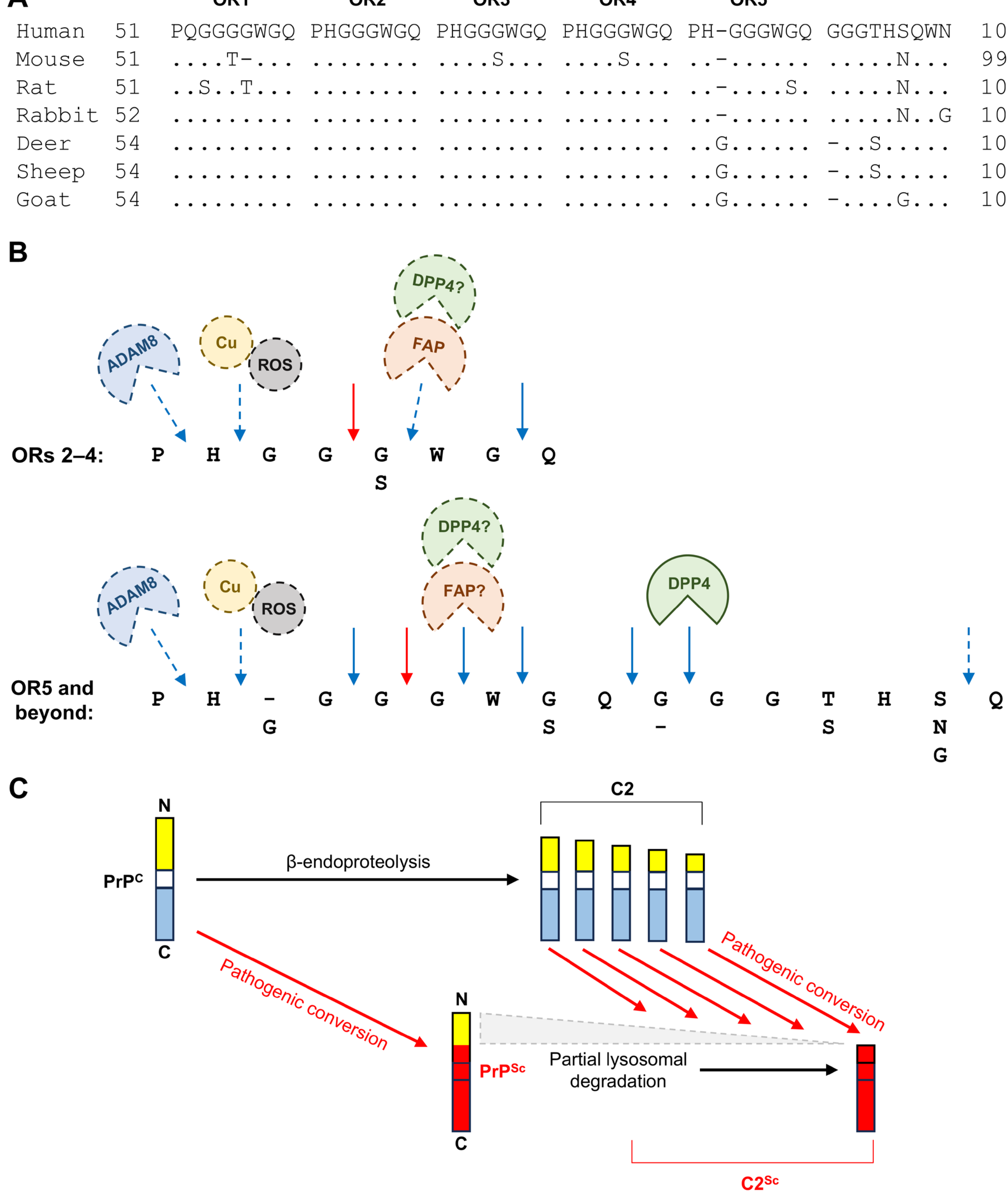

FIGURE 3 | Legend on next page.

brain homogenate did not identify any high-confidence fragments generated by β-cleavage of WT PrP^{C}, as might be expected from the relative rarity of this cleavage event in the brain (Gomez-Cardona et al. 2023). However, the authors did identify several medium-confidence, mostly post-glycine β-cleavage sites within ORs 3–5 as well as after Gly91, with the spectrum of fragments detected very similar to those found in the aforementioned study of ME7 scrapie-infected mouse brains. In addition to the publications already referred to, several studies reporting specific β-cleavage mechanisms have also determined potential cleavage sites and these findings will be considered in the sections below.

FIGURE 3 | β-Cleavage sites, mechanisms and potential $C2^{Sc}$-generating pathways. (A) Alignment of PrP^C sequences from various mammalian species (using NCBI blastp) covering the OR domain and flanking sequences. ORs 2–4 are almost identical among all the species, with a little more sequence variability found on either side. (B) Visualization of cleavage sites in the indicated regions (cleavages within the imperfect OR1 have not been reported). Sequence variation versus the human sequence is shown (for the species listed in panel A). Solid red arrows correspond to cleavages detected in prion-infected brain tissue (Hope et al. 1988), solid blue arrows to cleavages in normal brain tissue (Gomez-Cardona et al. 2023) and dashed blue arrows to cleavages in uninfected cells or in vitro assays (Castle et al. 2023; Mangé et al. 2004; McDonald et al. 2014). Potentially responsible β-PrPases are indicated based on data from in vitro cleavage assays (Castle et al. 2023; McDonald et al. 2014; Praus et al. 2003). DPP4-mediated cleavage at G/SQG↓GGT/S is the only proteolytic event conclusively detected both in brain tissue and in vitro (hence the solid outline for the protease). (C) The alternative pathways potentially leading to $C2^{Sc}$ generation: (1) attempted lysosomal degradation of FL PrP^{Sc} that progressively trims the protease-sensitive N-terminus, leaving the protease-resistant core intact and (2) conversion of C2 generated by β-endoproteolysis of normally folded PrP^C, possibly also followed by N-terminal trimming of the longer fragments in the lysosome.

3.2 | Candidate β-Cleavage Mechanisms

3.2.1 | Non-Enzymatic Cleavage by Reactive Oxygen Species in the Presence of Copper Ions

The initial study reporting a non-enzymatic form of PrP^C β-cleavage found that Cu^{2+} together with either H_2O_2 or superoxide (generated by the presence of xanthine and xanthine oxidase) induced rapid truncation of the anchorless PrP present in conditioned cell culture media, resulting in transient detection of an unstable ~6.5 kDa N-terminal fragment ('N2') (McMahon et al. 2001). These experiments were prompted by the knowledge that PrP^C can bind Cu^{2+} via the four His residues of its OR domain in addition to a nearby fifth site (His96/His111) (Brown et al. 1997; Burns et al. 2003; Hornshaw, McDermott, and Candy 1995; Jackson et al. 2001; Qin et al. 2002), as well as evidence that protein-bound copper can promote localized reactive oxygen species (ROS) generation via the Fenton reaction, potentially causing peptide bond hydrolysis in the immediate vicinity (Kim, Rhee, and Stadtman 1985). The theoretical underpinnings of Cu^{2+}/ROS-driven β-cleavage were fleshed out by a later study that incorporated insights from density functional theory and molecular dynamics, which suggested that PrP^C can reduce Cu^{2+} via electron transfer from the side chains of its OR-domain Trp residues (Pushie and Vogel 2009). The Cu^+ produced could then react with H_2O_2 to produce hydroxyl radicals that attack Gly residues within, and adjacent to, the OR domain, which is in line with the mainly post-glycine C2-generating events observed in healthy (Gomez-Cardona et al. 2023) and prion-infected mouse brains (Hope et al. 1988). However, a study confirming that hydroxyl radicals (produced by the combination of copper acetate and ascorbic acid) can promote β-cleavage of mouse recPrP in vitro found that cleavage events took place following OR His residues rather than after the neighbouring glycines (McDonald et al. 2014).

Although attractive in theory, the evidence for a Cu^{2+}/ROS-driven β-cleavage mechanism functioning in living cells is decidedly mixed. For example, exposing the retinal pigment epithelial cell line ARPE-19 to various agents known to increase ROS either directly or indirectly did not affect C2 levels, perhaps because the culture medium was not supplemented with extra Cu^{2+} (Chaudhary et al. 2021), whereas treatment with both H_2O_2 *and* Cu^{2+} did increase C2 production in SH-SY5Y human neuroblastoma cells (Watt et al. 2005). However, exposing CHO cells to H_2O_2 or stressing SH-SY5Y cells by incubation in a serum-free medium have both been shown to trigger β-cleavage without the need for experimental addition of Cu^{2+} (McMahon et al. 2001; Watt et al. 2005). Furthermore, although serum deprivation-induced C2 production in SH-SY5Y cells and constitutive β-cleavage in ARPE-19 cells were both blocked by the hydroxyl radical quencher dimethyl sulphoxide (Chaudhary et al. 2021; Watt et al. 2005), even relatively low concentrations of dimethyl sulphoxide can induce numerous changes to cellular physiology (Verheijen et al. 2019), so its effect on β-cleavage cannot be ascribed conclusively to ROS scavenging. The characterization of the Cu^{2+}/ROS-driven β-cleavage mechanism as solely a response to oxidative stress (Watt et al. 2005) also seems to be in conflict with the high levels of C2 production found in physiologically normal tissues of various types (Ashok et al. 2019, 2018; Ashok and Singh 2018; Asthana et al. 2017; Castle et al. 2023; Jimenez-Huete et al. 1998). Nonetheless, the N2 fragment of PrP^C has been reported to induce intracellular signalling processes that protect cells from oxidative stress (Haigh et al. 2009a, 2015b; Haigh, McGlade, and Collins 2015a).

3.2.2 | Calpains or Lysosomal Cathepsins

Initial experiments in the scrapie-infected SMB cell line showed that $C2^{Sc}$ levels were reduced following treatment with NH_4Cl, which prevents lysosomal acidification, or leupeptin, an inhibitor of lysosomal cathepsins (Caughey et al. 1991). The effects of these compounds were additive, although the combined treatment still permitted the production of intermediate PrP^{Sc} species 3–4 kDa smaller than FL PrP. Suggesting that the inhibitors may not have completely blocked lysosomal activity, the authors proposed that lysosomal enzymes progressively degrade the N-terminal domain of PrP^{Sc} in an attempt to destroy the misfolded protein (a process sometimes referred to as 'trimming'), but the protease-resistant core of residues ~90–231 is left unaffected. However, a later study found that more selective cathepsin inhibitors did not block $C2^{Sc}$ production by SMB cells, while calpain inhibitors were capable of doing so at relatively low concentrations; for example, calpain inhibitor IV reduced $C2^{Sc}$ levels with an IC_{50} of ~450 nM (Yadavalli et al. 2004). Overexpression of the endogenous calpain inhibitor calpastatin had a similar effect to the manufactured calpain inhibitors. The authors of this publication argued that rather than blocking lysosomal cathepsins, the effect of leupeptin in the original SMB cell line study was probably due to its ability to inhibit calpains, an

assertion supported by the literature on leupeptin (Saito and Nixon 1993). Nonetheless, another study found that high concentrations of calpain inhibitor III (MDL28170; ≥ 50 μM) did not affect PrP^{Sc} N-terminal trimming in infected cell lines, whereas cathepsin B or L was able to induce N-terminal truncation in in vitro digestion assays (Dron et al. 2010). The same article also demonstrated that lysosomal inhibition (with NH_4Cl alone or NH_4Cl plus leupeptin) completely blocked N-terminal truncation of PrP^{Sc} in mouse neuronal CAD5 cells infected with 22L or 139A mouse-adapted scrapie prions. Furthermore, a more recent study confirmed that inhibition of endosomal/lysosomal acidification by treatment with NH_4Cl or the V-ATPase inhibitor bafilomycin A1 increased intracellular FL PrP^{Sc} in prion-infected mouse neuroblastoma cells (Ribes et al. 2023). Therefore, cathepsins appear more likely to contribute to $C2^{Sc}$ production than calpains.

3.2.3 | ADAM8

As previously mentioned, in vivo data from ADAM8-null mice indicate that ADAM8 is a bona fide α-PrPase in muscle tissue (Liang et al. 2012), although whether it plays such a role in the brain is unclear. Interestingly, the study that confirmed that ADAM8 directly performs α-cleavage of recPrP in vitro also found that it displayed β-PrPase activity, cleaving recPrP within each of ORs 2–5 (McDonald et al. 2014). However, the particular cleavage event identified (GQP↓HGG) is distinct from those identified in the aforementioned mass spectrometry-based ex vivo studies of normal (Gomez-Cardona et al. 2023) and prion-infected mouse brains (Hope et al. 1988). Furthermore, given the absence of additional publications in this area, the link between ADAM8 and PrP^C β-cleavage in a cellular context remains uncertain.

3.2.4 | S9b Subfamily Peptidases

Recently, we reported that two serine proteases of the S9b peptidase subfamily, dipeptidyl peptidase-4 (DPP4) and fibroblast activation protein (FAP; a.k.a. prolyl endopeptidase FAP), perform β-cleavage of PrP^C (Castle et al. 2023). Edman sequencing of recPrP C-terminal fragments obtained from in vitro cleavage assays combined with analysis of immunoblotting band patterns in cell-based experiments revealed multiple cleavage sites for both proteases. In the case of FAP, cleavage events seemed to occur at GGG↓WGQ and GGS↓WGQ sequences in ORs 2–4, although the ambiguous identities of the first two residues in the Edman sequencing data mean that cleavages shifted one residue to either side cannot be ruled out. Recombinant DPP4 cleaved recPrP after Gly91, which lies just outside the OR domain, as well as inducing fragmentation within each of ORs 2–5, although the exact OR-domain cleavage sites could not be determined. Therefore, the identified DPP4 and FAP cleavage sites within PrP^C are broadly in line with the C-terminal fragments identified by ex vivo analysis of normal (Gomez-Cardona et al. 2023) and prion-infected mouse brains (Hope et al. 1988).

Cell-based DPP4 and FAP overexpression assays showed that neither the calpain inhibitor PD 151746 (10 μM) nor dimethyl sulphoxide (~141 mM) rescued the enhanced C2 production, suggesting that the increase in β-cleavage was not due to indirect effects relating to calpain inhibition or ROS production. Furthermore, unlike the other putative β-cleavage mechanisms, we obtained evidence of FAP-mediated β-cleavage occurring in vivo, as comparisons of WT and FAP-null mouse tissues showed that C2 levels relative to total PrP were reduced by ~40% in FAP-null adipose tissues, with smaller but statistically significant reductions observed in spleen and pancreas. Crucially, the extent of reduction in relative C2 levels positively correlated with the normal FAP expression level in the same tissue (in WT mice). As such, tissues normally lacking FAP expression were unaffected by *Fap* KO. This includes the brain, which indicates that FAP is unlikely to be involved in TSE pathogenesis. Nonetheless, several studies have hinted at physiological roles for β-cleavage, either in regulating iron homeostasis (Ashok and Singh 2018; Asthana et al. 2017) or in protecting cells from oxidative stress (Boufroura et al. 2021; Watt et al. 2005), so FAP may regulate these processes. Furthermore, although we could not confirm that DPP4 performs β-cleavage in vivo, DPP4 inhibition was effective at reducing PrP^{Sc} accumulation in prion-infected primary cerebellar glial cultures (Castle et al. 2023); we will return to this topic later.

3.2.5 | Summary of β-Cleavage Mechanisms

Figure 3B summarizes the multiple β-cleavage sites and proteolytic mechanisms identified from the relatively few studies on these topics. In the context of TSEs, lysosomal cathepsins may generate $C2^{Sc}$ by progressively truncating the N-terminal domain of PrP^{Sc}, but this mechanism lacks supporting in vivo data. In uninfected cells, the combination of Cu^{2+} and ROS such as H_2O_2 has been shown by several independent research groups to induce β-cleavage in cultured cells. However, under physiological conditions, it is unclear how an oxidative stress-regulated mechanism can explain both the prominent β-cleavage in certain tissues of the eye and in adipose, for example, as well as the much lower levels in tissues such as the brain. Our recent discovery that DPP4 and FAP are efficient inducers of PrP^C β-cleavage in cultured cells provides an alternative mechanism for C2 generation in healthy tissues, although we only obtained supporting in vivo evidence for the β-PrPase activity of FAP. Nonetheless, the effectiveness of DPP4 inhibition (but not FAP inhibition) at reducing PrP^{Sc} accumulation in prion-infected primary cerebellar glial cultures raises the possibility that DPP4 is normally blocked from performing β-cleavage of PrP^C, perhaps by endogenous serine protease inhibitors, but becomes active under pathological conditions. Further study into this possibility is needed, as is the investigation into whether β-cleavage by DPP4 or FAP and the Cu^{2+}/ROS-driven mechanism could somehow be related, particularly given the similarities in cleavage sites identified. For example, perhaps oxidative stress enhances DPP4/FAP-mediated β-cleavage.

3.3 | Subcellular Localization of β-Cleavage

As part of a study into the Cu^{2+}/ROS-driven β-cleavage mechanism, surface biotinylation experiments indicated that PrP^C β-cleavage takes place at the cell surface (Watt et al. 2005). Similarly, in our

recent publication, we found that the PrP^C GPI anchor was necessary for C2 production to occur (Castle et al. 2023). DPP4 and FAP are both type II membrane proteins, so β-cleavage occurring at the cell surface makes sense in this context. In contrast, the reduction in $C2^{Sc}$ caused by treating SMB cells with NH_4Cl suggests that N-terminal truncation of PrP^{Sc} can take place intracellularly in acidic compartments such as endosomes or lysosomes (Caughey et al. 1991; Dron et al. 2010), although we did not observe a clear effect of NH_4Cl applied at the same concentration in RML-infected primary cerebellar glial cultures (Castle et al. 2023).

3.4 | Relevance to the Pathogenesis of Neurodegenerative Diseases

Enhancing α-cleavage of PrP^C should reduce the accumulation of misfolded and aggregated PrP in prion-infected cells. In contrast, the reported β-cleavage sites are almost exclusively located on the N-terminal side of the protease-resistant core of PrP^{Sc}. Thus, C2 fragments generated by β-cleavage of normally folded PrP^C are capable of undergoing subsequent misfolding. But is β-cleavage neutral with regard to misfolding or is it pro-pathogenic? One potentially negative consequence of β-cleavage would be if it blocked a secondary α-cleavage to generate the C1 fragment, which was suggested by experiments in which C2 PrP (specifically residues 90–231) was overexpressed in HEK293 cells (Sunyach et al. 2007). However, analyses of human brain tissue have demonstrated that a longer post-mortem interval is associated with more C1 and less C2 (Jimenez-Huete et al. 1998), providing indirect evidence that C2 is a suitable substrate for α-cleavage. Another issue would be if C2 is more prone to misfolding than FL PrP^C, but relatively few studies have addressed this possibility directly. Interestingly, hamster recPrP lacking residues 34–94 was considerably less prone to template-mediated misfolding in vitro than FL recPrP (Lawson et al. 2001), but although this fragment approximates C2 in size, the deletion extends slightly further in the C-terminal direction than almost all reported β-cleavage sites, so the implications are unclear. In contrast, building upon earlier circular dichroism data suggesting that β-sheet formation within a recPrP fragment (residues 91–115) can be induced by Cu^{2+} (Jones et al. 2004), it has been proposed that Cu^{2+} binding involving His96 and/or His111 might trigger potentially pathogenic localized β-sheet formation that can then propagate to other regions of the protein – a process that would normally be inhibited by the OR domain (Pushie and Vogel 2009). Therefore, a C-terminal fragment containing His96 and His111 but lacking the OR domain (i.e., C2) may be more susceptible to misfolding than the FL protein (Pushie and Vogel 2009).

A finding indirectly implicating β-cleavage in TSE pathogenesis is the tendency for $C2^{Sc}$ to accumulate as disease progresses, with the clearest supporting data deriving from analyses in which cell or tissue lysates were not PK-treated in vitro but were deglycosylated with PNGase F. Overall, it appears that $C2^{Sc}$ is the dominant form of PrP^{Sc} in various cell line models of prion infection (Caughey et al. 1991; Dron et al. 2010) as well as in the spleen of mice with clinical-stage disease (Dron et al. 2010). Moreover, *de novo* infection of mouse neuroblastoma cells with exosomes containing PrP^{Sc} seeds generated detectable $C2^{Sc}$ within minutes, both intracellularly and at the cell surface, whereas FL PrP^{Sc} remained undetectable until at least 24 h after infection (Ribes et al. 2023). In the brain, the picture is less clear, with one study reporting minimal $C2^{Sc}$ in ovine PrP^C-expressing tg338 mice (high-susceptibility VRQ allele) infected with 127S scrapie, mouse PrP^C-overexpressing tga20 mice infected with 139A mouse-adapted scrapie and WT C57Bl/6 mice infected with 139A or 22L mouse-adapted scrapie strains (Dron et al. 2010). However, several other publications indicate that $C2^{Sc}$ is more abundant than FL PrP^{Sc} in the brain at the disease endpoint for the mouse-adapted scrapie strains RML and ME7, as well as the mouse-adapted bovine spongiform encephalopathy strain 301V (Mays et al. 2014; Watts et al. 2011; Yadavalli et al. 2004). Therefore, the extent of $C2^{Sc}$ accumulation may be strain-dependent. In this regard, it is interesting to note that a study of post-mortem human brain tissue found that brains with more PrP aggregates and more striking amyloid deposition tended to contain more $C2^{Sc}$ (Jimenez-Huete et al. 1998).

As alluded to earlier, $C2^{Sc}$ could be produced by two distinct mechanisms: (1) progressive removal of N-terminal residues from FL PrP^{Sc}, perhaps by lysosomal cathepsins, ultimately generating a protease-resistant core of residues ~90–231 and (2) β-endoproteolysis of normally folded FL PrP^C followed by template-mediated conversion of C2 to $C2^{Sc}$, perhaps also followed by some trimming of N-terminal residues given that β-endoproteolysis appears to occur at multiple positions in the OR domain (Figure 3C). Although in vivo confirmation is lacking, trimming by lysosomal cathepsins is an intuitively attractive possibility as one would expect lysosomes to be capable of degrading at least the protease-sensitive portion of FL PrP^{Sc}. However, the opportunity for therapeutic intervention here seems limited. Cathepsin inhibitors might reduce $C2^{Sc}$ production from FL PrP^{Sc}, but this would only lead to a consequent reduction in total PrP^{Sc} if $C2^{Sc}$ is better at driving misfolding of natively folded PrP^C than FL PrP^{Sc}. Experiments in mouse neuronal CAD5 cells indicate that this may not be the case, because lysosomal inhibition with NH_4Cl or NH_4Cl plus leupeptin completely eliminated $C2^{Sc}$ without affecting total PrP^{Sc} accumulation in cells infected with two different prion strains (Dron et al. 2010). In contrast, we found that the combined DPP4/FAP inhibitor linagliptin but not the FAP-specific inhibitor SP-13786 (both at 30 μM) reduced PrP^{Sc} levels by > 80% in primary cerebellar glial cultures infected with the mouse-adapted scrapie strain 22L in addition to reducing the sum of PK-resistant $C2^{Sc}$ plus PK-sensitive C2 relative to total PrP (Castle et al. 2023). Linagliptin also lowered PrP^{Sc} levels in RML-infected cultures of C2C12 cells differentiated into post-mitotic myotubes (Castle et al. 2023). Therefore, we have two studies, one in which reducing $C2^{Sc}$ levels was highly effective at reducing total PrP^{Sc} accumulation and one in which completely eliminating $C2^{Sc}$ had no effect on total PrP^{Sc} accumulation. Perhaps this discrepancy is underpinned by differences in the rate at which normally folded FL PrP^C undergoes β-cleavage, thereby changing the proportion of $C2^{Sc}$ generated by misfolding of pre-existing C2 and the proportion deriving from N-terminal truncation of already misfolded FL PrP^{Sc}. As previously discussed, β-cleavage prevalence varies dramatically among different cell types, with C2 found at low but detectable levels (relative to total PrP^C) in uninfected brain tissue. Given that DPP4 KO in uninfected mice did not significantly reduce C2 levels in the brain, it seems contradictory that DPP4 inhibition had a beneficial effect in the prion-infected primary cerebellar glial cultures (Castle et al. 2023). However, is it possible that DPP4 activity increases in the prion-infected brain? Interestingly, upregulation of astrocytic DPP4 expression by neuroinflammation has been

demonstrated in a rat model (Kiraly et al. 2018), and astrocytes appear to be converted to a pro-inflammatory phenotype during the early stages of TSE pathogenesis (Hwang et al. 2009; Majer et al. 2019). Moreover, pro-inflammatory microglia in human iPSC-derived neuron–microglia co-cultures are reported to shed DPP4 from their cell surface into the culture medium (Vahsen et al. 2023). An increase in glial DPP4 expression offers a potential explanation for why β-cleavage of normally folded PrP^C may be more prominent in infected primary cerebellar glial cultures (and perhaps also the brain) than in neuronal models lacking glial cells (e.g., CAD5 cells). In addition to changes in DPP4 expression, it is possible that DPP4 activity is enhanced by reduced levels of endogenous serine protease inhibitors. Furthermore, prion infection may also compromise antioxidant systems leading to increased oxidative stress (Milhavet et al. 2000), which could plausibly result in enhanced β-cleavage via the Cu^{2+}/ROS mechanism. Thus, more studies are needed to clarify the potential anti-prion effects of inhibiting PrP^C β-cleavage, including (1) determining conclusively whether or not C2 is more prone to misfolding than FL PrP^C; (2) assessing whether DPP4 inhibition and/or antioxidant compounds reduce total PrP^{Sc} accumulation in a wider variety of models, particularly given that mouse DPP4 appears to be a more effective β-PrPase than human DPP4 (Castle et al. 2023) and (3) testing whether DPP4 KO increases incubation times in TSE-infected mice.

4 | Brief Notes on Other Prion Protein Fragmentation Events

4.1 | Shedding of PrP^C by Cleavage Adjacent to the GPI Anchor

In this review, we have focused on α- and β-endoproteolytic cleavages of PrP due to their apparent opposing roles in TSE pathogenesis. However, additional PrP fragmentation events have been identified. For example, mouse and human forms of PrP can be shed from the cell surface via ADAM10-mediated cleavages at Gly228↓Arg229 (Taylor et al. 2009) and Tyr226↓Gln227 (Song et al. 2024), respectively. In a similar manner to N1, shed PrP may interact with Aβ oligomers in the extracellular space, reducing their ability to bind to receptors on the cell surface and induce toxicity through hyperactivation of Fyn kinase signalling (Jarosz-Griffiths et al. 2019). Furthermore, loss of PrP^C shedding due to *Adam10* KO increases cell-surface PrP^C levels, leading to greater PrP^{Sc} formation in mice infected with RML scrapie prions and reduced disease incubation times (Altmeppen et al. 2015). Conversely, the shedding of PrP^{Sc} may enhance the spread of prion pathology within the brain (Altmeppen et al. 2015). For more information on PrP shedding, we draw the reader's attention to recent review articles on this topic (Matamoros-Angles et al. 2023; Mohammadi et al. 2023).

4.2 | Gamma-Cleavage of PrP^C in a C-Terminal Region

During experiments to develop a cell-surface protein labelling technique, a ~9 kDa novel fragment of PrP^C was discovered (Taguchi et al. 2009). Although not the focus of their study, the authors termed the fragment 'C3' and found that the broad-spectrum cysteine protease inhibitor E64 reduced its production. In a separate later study, analysis of lysates from various cell lines using an antibody with a far C-terminal epitope (residues ~214–230) revealed an unexpected 6–7 kDa C-terminal fragment that the authors also called C3, while naming the proteolytic event responsible as γ-cleavage (Lewis et al. 2016). The 6–7 kDa C3 fragment appeared to be produced in the Golgi or trans-Golgi network, although it was unclear whether this occurred during secretion or after recycling from the cell surface or both (Lewis et al. 2016). Although largely absent from healthy human brain tissue, the 6–7 kDa C3 has been detected in bank vole brain homogenates and at lower levels in the brains of transgenic mice overexpressing PrP^C from various species, while a protease-resistant form of the fragment was prominent in human CJD brain tissue (Lewis et al. 2016; Vanni et al. 2023). Based on its size and lack of glycosylation, γ-cleavage is thought to occur on the C-terminal side of the second and final N-glycosylation site (Asn197 in humans) (Vanni et al. 2023). Interestingly, the report that introduced the term γ-cleavage also refers to a slightly longer fragment, C3′, that was detected after cell lysates were deglycosylated with PNGase F and was additionally found in human CJD brain tissue. This potential precursor fragment is fully glycosylated and the cleavage site has been narrowed down by antibody epitope mapping to residues ~170–181 (Vanni et al. 2023). Given the variability in SDS-PAGE migration and the accuracy of protein standards, the 9 kDa 'C3' of the original study may be equivalent to the 6–7 kDa C3 or the C3′ fragment identified later, although production of 6–7 kDa C3 was suppressed by a matrix metalloproteinase inhibitor but not by treatment with E64, perhaps indicating a different origin. Further investigations are needed to identify the protease(s) responsible for γ-cleavage and determine its role (if any) in TSE pathogenesis.

4.3 | Generation of Atypical Pathological PrP Fragments in Gerstmann–Sträussler–Scheinker Syndrome

Gerstmann–Sträussler–Scheinker syndrome (GSS) is a familial prion disorder caused by several different mutations within the *PRNP* open-reading frame that is clinically distinct from inherited forms of CJD (Mastrianni 2010). GSS prions are characterized by atypical PK-resistant cores of 6–8 kDa with C-termini ranging from residues ~147–153, sometimes co-existing with PrP^{Sc} containing a PK-resistant core of the standard 19–21 kDa size when unglycosylated (Ghetti, Piccardo, and Zanusso 2018). Mouse models of GSS transmission or spontaneous disease have also consistently identified 6–8 kDa PK-resistant PrP^{Sc} (Mercer et al. 2018; Pirisinu et al. 2016; Vanni et al. 2020). Interestingly, use of thermolysin, a protease that degrades PrP^C while leaving the PK-sensitive N-terminal residues of PrP^{Sc} intact (Owen et al. 2007a), revealed that ~16 kDa, unglycosylated, thermolysin-resistant PrP with ragged C-termini in the vicinity of residue ~155 may be a precursor to the signature 6–8 kDa PK-resistant PrP^{Sc} (Mercer et al. 2018). Enrichment of abnormal PrP from brain tissue via phosphotungstic acid precipitation showed that the ~16 kDa form and, to a lesser extent, the ~8 kDa form are present even without in vitro protease digestion, indicating that they can be generated by endogenous protease activity in vivo (Mercer et al. 2018). C-terminal truncation/trimming due to aborted lysosomal degradation seems an unlikely explanation for the ~16 kDa fragments, given that the N-terminal residues

are PK-sensitive and, therefore, should be degradable. Instead, GSS-linked mutant forms of PrP probably undergo an endoproteolytic cleavage around residue 155 either before or after undergoing pathogenic conformational change; notably, the cleavage position does not match a γ-cleavage event (see above). Later, the ~16 kDa product may undergo N-terminal trimming in the lysosome in a manner akin to $C2^{Sc}$ generation from FL PrP^{Sc}. Further investigations into the identity of the protease responsible for endoproteolysis around residue 155 are warranted, given that this cleavage event could plausibly be a trigger for misfolding of the resulting N-terminal fragment in GSS.

4.4 | Generation of PrP^C Neuropeptides?

In our recent publication linking S9b subfamily peptidases to β-endoproteolysis of PrP^C, we also showed that recombinant DPP4 efficiently cleaved recPrP close to its N-terminus at Pro39↓Gly40 (Castle et al. 2023). Recombinant FAP cleaved recPrP similarly, but the exact position could not be determined. Using the N-terminal antibody EB8, which has an epitope in the 26–34 region (Didonna et al. 2015), we were unable to detect the N-terminal fragments that should have been produced. Nonetheless, given that cleavage at Pro39↓Gly40 occurred at a lower DPP4 concentration than β-endoproteolysis in the OR domain, we believe the possibility that short N-terminal peptides can be generated from cell-surface PrP^C is worthy of further investigation, particularly given what is already known about the PrP^C N-terminus. For example, residues 23–41 are almost completely conserved among humans, ruminants and rodents (Figure 4), which is suggestive of an important function. One established role associated specifically with the cluster of mostly positively charged residues (KKRPK) at the far N-terminus of mature PrP^C is activating the G-protein-couple receptor Adgrg6, thereby triggering signalling pathways to maintain the myelin sheath around peripheral nerves (Kuffer et al. 2016). The same cluster of residues has been attributed cell-penetrative properties akin to those of human immunodeficiency virus Tat protein (Wadia et al. 2008) and may have membrane pore-forming abilities (Solomon et al. 2011; Westergard, Turnbaugh, and Harris 2011b). The membrane pore-forming property of the charged cluster is reportedly suppressed by cis interactions between the N- and C-terminal domains (McDonald et al. 2019; Roseman et al. 2020; Schilling et al. 2023, 2020), and certain amino acid changes to PrP^C associated with inherited TSEs may be neurotoxic due to disruption of this *cis* interaction, at least in part (Schilling et al. 2020). However, a beneficial role for the far N-terminus has also been proposed based on the findings that it can lethally damage the membranes of microbes such as *Escherichia coli*, *Staphylococcus aureus* and the fungal pathogen *Candida parapsiliosis* in vitro (Pasupuleti et al. 2009). There is an intriguing parallel here with research in the AD field demonstrating the antimicrobial abilities of Aβ peptides against bacterial, fungal and viral pathogens in the brain (Eimer et al. 2018; Kumar et al. 2016). Based on these findings, one hypothesis is that Aβ levels increase with age as a response to more frequent encroachment of pathogens into the brain due to less effective immune function in the periphery, with chronically high Aβ production triggering the accumulation of Aβ aggregates and, ultimately, leading to the clinical features of AD (Vojtechova et al. 2022). Although speculative, this hypothesis offers an explanation for the association between regular vaccination (e.g., seasonal influenza vaccines) and a lower risk of dementia in older adults (Wu et al. 2022). Could similar processes be at work for PrP^C and could they play a role in TSE pathogenesis? Currently, we do not know whether DPP4/FAP can generate short PrP N-terminal fragments in living cells, but we have previously mentioned that neuroinflammation appears to increase astrocytic DPP4 expression and DPP4 shedding by microglia (Kiraly et al. 2018; Vahsen et al. 2023). Therefore, if it does occur in vivo, DPP4-mediated production of PrP^{23-39} could increase and become chronic under conditions of neuroinflammation, potentially damaging neuronal membranes and causing neurotoxicity. Further studies will be needed to test this possibility, including whether this putative mechanism could explain some of the neuronal death that occurs in TSEs.

5 | Closing Remarks

In this review, we have outlined the current understanding of PrP^C endoproteolytic processing, focusing mainly on α- and β-endoproteolysis. In terms of α-endoproteolysis, recent evidence indicates that there may be a group of distinct fragmentation

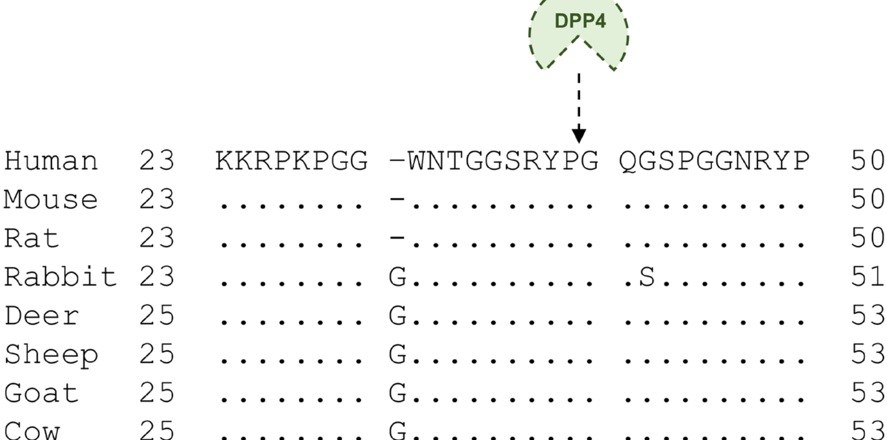

FIGURE 4 | Alignment of PrP^C N-terminal sequences from various mammalian species. The alignment (obtained using NCBI blastp) demonstrates the complete sequence identity of the PrP^C N-terminal region between humans and rodents, with only minimal changes found in other mammalian species. The position of DPP4-mediated cleavage demonstrated in vitro is indicated.

events occurring at multiple sites within residues 109–121, although cleavage at a unique initial site followed by variable aminopeptidase trimming cannot be ruled out. Several candidate α-PrPases have been identified, underlining the likely redundancy in this process, but the two proteases most convincingly linked to α-cleavage, ADAM8 and plasmin, are still not proven to perform this function in neurons. There is less doubt over the role of α-endoproteolysis in the pathogenesis of TSEs, which is almost certainly a protective one. Thus, clarifying the relative contributions of (potentially) several different α-PrPases to neuronal C1 production will be important to build on the promising results from cell line models in which α-cleavage has been enhanced. The therapeutic interventions that may follow could complement approaches to reduce overall PrP levels that are also being developed. Upregulating neuronal α-cleavage may even have benefits in other neurodegeneration disorders, particularly AD.

Regarding β-endoproteolysis, a major issue in the field has been reconciling the early discovery of N-terminally truncated PrP^{Sc} in prion disease models with the later identification of very similarly sized C-terminal products of cleavages in and around the OR domain that can be abundant in healthy tissues of many types. Although N-terminal trimming of PrP^{Sc} in the lysosome probably happens to some extent, a key question yet to be fully answered is whether β-cleavage of normally folded PrP^{C} followed by C2 misfolding contributes significantly to the increase in $C2^{Sc}$ levels relative to FL PrP^{Sc} that tends to occur as disease progresses. Nonetheless, it is striking that both DPP4- and Cu^{2+} plus ROS-mediated β-cleavage mechanisms could plausibly be upregulated under pathological conditions in the brain, while DPP4 inhibition has already been shown to reduce PrP^{Sc} levels in prion-infected primary glial cultures. Crucial next steps include testing whether inhibiting β-cleavage proves similarly effective in a wider variety of models, particularly in cultured human neurons or organoids in which DPP4 may be a less effective β-PrPase than in mouse models, as well as investigating whether a connection between DPP4/FAP- and Cu^{2+} plus ROS-mediated β-cleavage mechanisms can be found. It would also be interesting to clarify whether short N-terminal fragments ($\sim PrP^{23-39}$) are generated by DPP4 and/or FAP in neural cells and, if so, what are the physiological and/or pathological consequences of their production. Thus, in spite of considerable progress in the decades since PrP fragmentation was first recognized, we still need answers to several key mechanistic questions to arrive at a definitive description of the complex nature of PrP^{C} endoproteolysis and its contribution to TSE pathogenesis.

Author Contributions

Andrew R. Castle: conceptualization, writing – original draft, writing – review and editing. **David Westaway:** writing – review and editing, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing are not applicable since no new data were generated for this manuscript.

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