CERVID PRION PROTEIN POLYMORPHISMS MODULATE THE DIVERSITY OF CHRONIC WASTING DISEASE PRION STRAINS

By

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ABSTRACT

Chronic wasting disease (CWD) is a contagious prion disease spreading and emerging in wild and captive *Cervidae* species worldwide. Prion diseases are fatal neurodegenerative disorders occurring in various mammalian species including deer, elk, sheep, cattle and humans. Central to prion pathogenesis is the *PRNP* gene, which encodes the cellular prion protein (PrP^C), a membrane glycoprotein that is primarily expressed in the brain. The pathogenesis of prion diseases involves the misfolding of PrP^C into self-replicating, strain-encoding PrP^{Sc} (PrP^{CWD} for cervid infections) conformers termed "prions". Single PrP^C amino acid polymorphisms can influence aspects of the disease, including susceptibility, clinical presentation and pathological phenotypes. In cervids, transmission of CWD occurs within and between species expressing *PRNP* allelic variants. A potential consequence of prion transmission between hosts with different PrP^C primary structures is the emergence of CWD strains with novel transmission properties.

These studies characterize the biological effects of CWD propagation in deer expressing different PrP^C molecules:

I) PrP^C amino acid polymorphisms H95 and S96 subjected CWD prions to selective propagation barriers that modified the spectra of PrP^{CWD} conformers.

II) Transmission of PrP^{CWD} conformational variants resulted in the emergence of a novel CWD strain that was selected in concert with the host recipient PrP^C.

III) CWD transmission between deer expressing different PrP^C primary structures expanded the host range of CWD.

These results suggest that circulating CWD strains would diversify as transmission between different cervid *PRNP* genotypes occurs. Understanding the effects of PrP^C polymorphisms on the properties of CWD strains could help identify key events in their replication and spread.

PREFACE

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Etiology and Pathogenesis of Prion Diseases", AUP00000914.

Chapter 2 of this thesis is my original work with the collaboration of Dr. Jiri Safar, Tracy Haldiman and Chae Kim from the National Prion Disease Pathology Surveillance Center, Case Western Reserve University, USA. Trace Haldiman and Chae Kim performed conformation dependent immunoassays and conformation stability assays. The rest of the data presented in this chapter is my original work.

Chapter 3 of this thesis is my original work with supporting animal breeding and genotyping by Chiye Kim and histological processing by Hristina Gapeshina and Dr Nathalie Daude. This chapter has been published in the Journal of Virology and is reproduce here:

Duque Velásquez C, Kim C, Herbst A, Daude N, Garza MC, Wille H, Aiken J & McKenzie D. Deer Prion Proteins Modulate the Emergence and Adaptation of Chronic Wasting Disease Strains. Journal of Virology 89: 12362-12373. Copyright © 2015 Duque Velásquez et al. American Society for Microbiology.

Chapter 4 of this thesis was developed and co-written in collaboration with Dr. Allen Herbst and Elizabeth Triscott and is currently under revision for publication.

Herbst A*, Duque Velásquez C*, Triscott E, Aiken J & McKenzie D. Emergent strains expand the host range of chronic wasting disease. Emerging Infectious Diseases. Submitted on Sept 06, 2016EID-16-1474

Dr Allen Herbst and I initiated the transmission experiments in C57BL/6 mice and hamsters presented in Chapter 4. We performed the animal scoring, tissue collection, analysis of incubation periods and analysis of C57BL/6 mice brains by western blot. Elizabeth Triscott and I analyzed the hamster brains by western blot.

The literature review presented in Chapter 1 and the concluding analysis in Chapter 5 are my original work.

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KEYWORDS

Cervids – General term to describe species of the mammalian *Cervidae* family

PrP^C – The normal cellular prion protein, susceptible to PK digestion

PRNP – Mammalian gene encoding PrP^C

Prions – The self-propagating disease associated conformers of PrP^C

PrP^{Sc} – The abnormal amyloidogenic, disease-associated conformer of PrP^C

PrP^{CWD} – PrP^{Sc} during cervid infections

PrP^{CWD} aggregates – PrP^{CWD} assemblies or deposits accumulated brain

PK-res PrP^{CWD} – Abnormal prion protein resistant to proteinase K

PK-sen PrP^{CWD} – Abnormal prion protein sensitive to proteinase K

Conformation –The three dimensional structure of a protein

CHAPTER 1.

INTRODUCTION TO PRION DISEASES

1.1. Introduction

Transmissible spongiform encephalopathies (TSE) or prion diseases are fatal neurodegenerative disorders of various mammalian species. Animal prion diseases include chronic wasting disease (CWD) in cervids, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE also known as "mad cow disease") and transmissible mink encephalopathy (TME). In humans, prion diseases occur most commonly as age-associated disorders such as sporadic Creutzfeldt-Jakob disease (sCJD) or as genetic diseases; Gerstmann-Straüsler-Scheinker (GSS) and fatal familial insomnia (FFI) (reviewed by Geschwind, 2015). Inadvertent exposure or ingestion (cannibalism) of TSE-contaminated tissues from persons with prion disease resulted in epidemics of iatrogenic Creutzfeldt-Jakob disease (iCJD) and *Kuru* (Brown et al., 2012; Gajdusek and Zigas., 1957). Prion diseases can be transmissible between different host species. BSE infection was demonstrated to be the cause of variant Creutzfeldt-Jakob disease (vCJD) in humans (Bruce et al., 1997). BSE is transmissible to goats, felines and various zoo animals (Eloit et al., 2005; Spiropoulos et al., 2011; Sigurdson and Miller, 2003).

Neuropathogenesis of prion diseases occurs over extended asymptomatic periods and depends on the misfolding of host-encoded (*PRNP*) cellular prion proteins (PrP^C) into infectious, self-propagating and aggregative conformations (PrP^{Sc}) termed "prions" (for proteinaceous infectious particles) (Prusiner 1982a; Bueler et al., 1993). Prion diseases manifest as progressive neurodegenerative disorders characterized by clinical and pathological features that depend on host-pathogen interactions. The brain pathology of affected hosts involves spongiform

degeneration, neuronal loss, gliosis, and accumulation of PrP^{Sc} in the form of aggregates (Figure

1.1).

Figure 1.1. Histopathology of chronic wasting disease in the obex of white-tailed deer**.** A) Spongiform change as evaluated by hematoxylin & eosin. B) PrP^{CWD} aggregates revealed by immunohistochemistry with anti-PrP antibody BAR224. C) Astrogliosis visualized by immunohistochemistry with anti-GFAP antibody. D-F) Obex tissues from uninfected deer. Delimited areas correspond to higher magnification panels. Black bar = 2.5 mm.
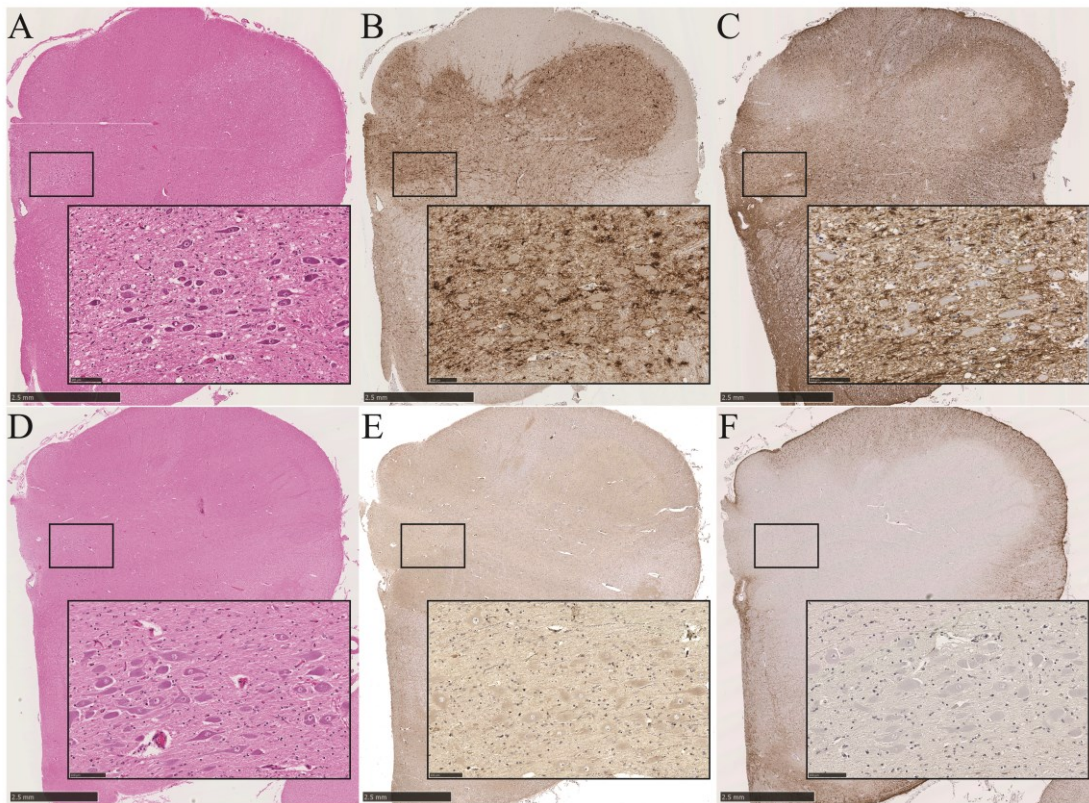
Variation in neuropathology and disease presentation within host species can occur in concert with different prion strains (Bruce et al., 1991; Bessen and Marsh, 1992a). In the absence of pathogen-specific nucleic acids, the identity of prion strains is thought to be encoded and perpetuated by their PrP^{Sc} structure or conformation (Marsh and Bessen 1992b; Hill et al., 1997; Safar et al., 2015).

Following exposure to prions, the host susceptibility to develop prion disease, the clinical presentation, and the neuropathology are regulated by the interaction between the host PrP^C primary structure and the invading prion strain (Hill et al., 1997; Wadsworth et al., 2004). Transfer of prion strains between hosts expressing different PrP^C primary structures can introduce permanent or reversible modifications resulting in the emergence of strains with novel transmission properties (Kimberlin et al., 1987; 1989; Bartz et al., 1998; Bartz et al., 2000). Selection of strains from a mixture can also occur following transmission between species expressing different PrP^C molecules (Bruce, 1993).

CWD epidemics in wild or captive cervids from North America, Norway or South Korea can involve prion transmission within and between various species and across distinct *PRNP* genotypes that encode PrP^C amino acid polymorphisms (reviewed in Robinson et al., 2012b; Benestad et al., 2016). Evidence for the existence of different CWD strains keeps increasing. The mechanism of strain variation and the effects of prion propagation in cervids expressing different PrP^C primary structures remains to be clarified (Bartz et al., 1998; Raymond et al., 2007; Angers et al., 2010; Duque Velásquez et al., 2015).

1.2 Etiology: From slow viruses to prions

The nature of the causal agent and the pathogenesis of prion diseases are complex research questions studied for more than two centuries and that still require investigation. The study of scrapie in sheep dates further back than any of the other TSEs. It was initially claimed by Roche-Lubin (1848) that scrapie resulted from exposure to lighting or sexual excess (reviewed in Pattison, 1972). The experimental transmission by inoculation of nervous tissues from affected sheep demonstrated that a replicative pathogen, perhaps a virus, was the infectious agent behind this epizootic (Cuille and Chelle, 1936).

Scrapie-contaminated "looping-ill" vaccines developed at Moredun Research Institute in Edinburgh and administered to sheep during 1935, inadvertently confirmed the transmissible nature of scrapie (Gordon, 1946; reviewed by Pattison, 1972). The tolerance of the scrapie agent to the inactivation conditions of these vaccines (formalin 0.35% w/v for three months) raised doubts about the viral nature of the scrapie agent.

Wilson (also at Moredun Institute) subsequently confirmed scrapie was filterable and transmissible to sheep (Wilson, Anderson and Smith, 1950). Wilson's work, mostly unpublished, revealed the remarkable tolerance of the scrapie agent to inactivation by heat (at 100°C for 30 minutes), 3% formalin, 5% chloroform or 4% phenol (each at 37°C for two weeks). Most importantly, he first showed the resistance of scrapie homogenates to inactivation with UV-irradiation, which brought into question the viral nature of the scrapie (reviewed by Pattison, 1972).

The inability to define the biological properties of the scrapie agent, the long incubation periods, the protracted disease progression and the communicable nature, led Sigurdsson (1954) to suggest scrapie was a "slow virus" disease; a category he proposed to differentiate from acute and chronic diseases caused by "conventional" viruses (reviewed by Pattison, 1972). To date, no such biological entity has been associated with TSEs affected hosts.

The characterization of the scrapie agent properties was complemented by the demonstration of its transmissibility into mice and hamsters, which resulted in shorter and less variable incubation periods (Chandler, 1961; Zlotnik and Rennie, 1965). Infection in rodent models favored a more precise and practical quantification of the infectious doses that survived after physical and chemical denaturing procedures.

Despite initial thermal resistance studies showing that scrapie brain preparations remain infectious to sheep after boiling for 8 hours at 99.5°C, proper estimation of the effects this treatment had on the agent infectivity could not be practically ascertained (Stamp et al., 1959). Hunter and Millson (1964) would later reveal that despite a considerable loss of infectivity, traces of the scrapie agent could still be detected by mouse bioassay after autoclaving brain preparations at 100°C for 1 hour and at 118°C for 10 minutes.

Scrapie has also been shown to resist various chemical treatments. Pattison (1965) expanded on previous observations by demonstrating the scrapie agent could be transmitted to goats and mice after treatment with 12% formalin for 28 months. Treatment with chemical agents that were known to have virucidal properties offered similar conclusions (Stamp et al., 1959; Haigh and Clarke, 1968); exposure of scrapie to ß-propiolactone had little effect on the scrapie agent but

completely inactivated the yellow fever virus (Haigh and Clarke, 1968). The resistance of scrapie to high doses of UV-irradiation provided strong support against the viral nature of the transmissible agent and suggested that exogenous nucleic acids are not required for its replication (Alper et al., 1967).

1.3 Prion hypothesis

Encouraged by the apparent nucleic acid independent-replication of scrapie and its unusual properties, various hypotheses emerged regarding its composition. These included replicative polysaccharides (Field, 1967), a membrane linkage-substance (Gibbons and Hunter, 1967), a hypothetical nucleic acid 'viro' protected by a host protein (Dickinson and Outram, 1979) and the self-replicating protein hypothesis (Griffith, 1967). Further studies showed the scrapie agent tolerated treatment with carbohydrases, lipases, and nucleases, but was highly susceptible to inactivation by proteases and protein denaturing chemicals (i.e., phenol, SDS, guanidine) (Prusiner et al., 1980; Prusiner, 1982a). In addition to these properties, hydrophobic proteins were isolated in brain preparations from terminally ill rodents but not from uninfected ones (Prusiner et al., 1981).

The accumulated evidence supported the self-replicating protein hypothesis leading Stanley Prusiner (1982a) to propose the scrapie agent was a proteinaceous particle and to coin the term "prion". Prusiner also remarked on William Hadlow's association between the transmissible and neuropathological features of Kuru and CJD with those of scrapie. Hadlow, Prusiner, and others

(1980a) had previously transmitted CJD into goats, suggesting it was a disease related to scrapie (Hadlow et al., 1980a; Hadlow, 1995; Gadjusek, Gibbs and Alper, 1966).

The identification and isolation of a protein fraction that co-purified with infectivity and was not present in unaffected animals resulted from studies using brains from scrapie-infected hamsters and ultracentrifugation in sucrose gradients (Prusiner et al., 1982b; Bolton, McKinley and Prusiner, 1982). The relative resistance of prions to proteinase K treatment, a step in the purification protocol, yielded a protein fraction of approximately 27 to 30 kiloDaltons when resolved on SDS-polyacrylamide gels (SDS-PAGE) (Bolton, McKinley and Prusiner, 1982). The association of the 27-30 kDa prion protein (PrP27-30) with infected but not normal hamsters, led to the interpretations that this protein was a structural component of the scrapie agent or was produced in response to the infection (Bolton, McKinley and Prusiner, 1982).

Purified peptides derived from the PrP27-30 fraction were sequenced and used to generate hybridization oligonucleotides that later served to screen a cDNA library prepared from infected hamsters (Oesch et al., 1985). The identified clone and the predicted peptide sequence indicated that PrP27-30 was derived from a larger protein (Oesch et al., 1985). Southern and northern blot analysis of hamster genomic DNA and poly (A) RNA prepared from infected and uninfected animals confirmed that PrP27-30 was encoded by a cellular gene (Oesch et al., 1985). A similar gene (*Prnp*) was later identified in mouse (Carlson et al., 1986) and humans (*PRNP*) (Kretzschmar et al., 1986). Immunoblot analysis of control and infected brain preparations revealed that cellular PrP (PrP^C) in the uninfected animals was susceptible to proteolysis, while

(PrP^{Sc}) in the infected hamsters is resistant to proteinase K (PK-res PrP^{Sc}) yielding the PrP27-30 fragments (Oesch et al., 1985).

Based on the cumulative evidence, the leading hypothesis, commonly known as the "protein-only hypothesis", states that prion diseases involve the misfolding of PrP^{C} into the pathogenic conformation (PrP^{Sc}; Sc for scrapie: Prusiner, 1997). Additional supporting evidence of this hypothesis included the discovery of human *PRNP* mutations associated with familial predisposition to prion disease (Hsiao et al., 1989; 1991). Transgenic expression in mice of the Gerstmann-Sträussler syndrome-associated L102P mutation induced spontaneous prion disease and characteristic neuropathology (Hsiao et al., 1990). Transmission studies also provided support for the protein-only hypothesis. Hamster scrapie prions transmit poorly into mice and vice versa, however, transgenic expression of hamster PrP^{C} in mice resulted in prion disease after short incubation periods and production of hamster-specific infectivity (Scott et al., 1989). In addition, mice for which the expression of PrP^{C} was genetically ablated were refractory to infection and failed to sustain prion replication (Büeler et al., 1993).

Skeptics of the protein-only-hypothesis insist on the existence of an unidentified nucleic acid or co-factor molecules. Their criticism rests on the existence of multiple strains (in the absence of nucleic acids) or transmission studies interpreted as the lack of correlation between infectivity and PrP^{Sc} (Lasmézas et al., 1997; Botsios and Manuelidis, 2016). Nonetheless, more data keeps tilting the balance towards the protein-only hypothesis. Various authors have demonstrated the ability of recombinantly expressed PrP to form aggregates that can trigger prion disease pathology and propagate different strain characteristics upon their passage in rodents (Colby et al., 2009;

Makarava et al., 2010). Most recently, amyloid fibrils composed of recombinant N-terminal fragments PrP (23-144) were shown to be transmissible to transgenic mice (Choi et al., 2016).

1.4 Prion diseases in non-human mammalian species

1.4.1. Scrapie is a worldwide naturally occurring TSE of sheep (*Ovis aries*) and goats (*Capra sp*). Questionable exceptions include countries that have implemented strict surveillance and eradication programs (Hunter and Cairns, 1998). Scrapie is the prototypic prion disease and has been recognized in Britain and Germany's flocks for approximately 267 and 285 years respectively (reviewed by Fast and Groschup, 2013).

Scrapie occurrence in flocks is observed most frequently in sheep between 2 to 5 years old. However, a smaller proportion of cases occur in older (over six years old) animals and rarely before two years (Simmons et al., 2009). Once neurological signs become evident the disease worsens progressively; motor dysfunction, behavioral and sense alterations occur before recumbency and death. Aggressiveness, biting, and other symptoms have been recorded in scrapie-affected goats compared to sheep (Capucchio et al., 2001). Different neurological syndromes or disease presentations can occur; these include pruritic and non-pruritic forms (Pattison and Milson 1961; Konold and Phelan, 2014; Capucchio et al., 2001).

The natural spread of scrapie occurs by direct contact between infected and unexposed animals. Transmission from mother to offspring or by exposure to scrapie-contaminated paddocks also

occurs (Ryder et al., 2004; Foster et al., 2006; Konold et al., 2013; Schneider et al., 2015). The introduction of sheep from scrapie-free regions to habitats that held infected flocks several years before repopulation, resulted in transmission of scrapie (Georgsson, Sigurdarson and Brown, 2006). Detection of scrapie infectivity in placenta, kidneys and saliva, indicates extraneural infection contributes to environmental contamination with scrapie (Sisó et al., 2008; Schneider et al., 2015). The first accumulation of scrapie infectivity and PrP^{Sc} in the tonsils, the jejunum and ileum (gut-associated lymphoid tissues) and the corresponding lymph nodes that drain these tissues indicates infections occur by ingestion of the scrapie agent (Van Keulen, Vromans and Zijderveld, 2002).

1.4.2. Bovine spongiform encephalopathy best exemplifies the risks prion diseases can pose to animal and human health. First identified in Great Britain's cattle during the late 1980's, the incidence of BSE rose drastically, becoming an epizootic that later reached other countries (Wells at al., 1987; Brown et al., 2001). BSE is a foodborne zoonotic prion disease and, although the source of infection remains unknown, feeds contaminated with cattle-derived proteins enhanced its spread in Great Britain and other countries. The introduction of bans on the usage of ruminant-derived feed was followed by a reduction of BSE cases (Brown et al., 2001).

Distinct BSE presentations occur, cattle affected with the 'classical' form of BSE (C-BSE) have incubation periods that range between 2 and 8 years. The clinical presentation may include hind limb ataxia and incoordination, abnormal posture, hyperresponsiveness and behavioral alterations such aggressiveness, nervousness or apprehension (Wells et al., 1987; Konold et al., 2012).

Atypical BSE presentations are less understood with cases being primarily identified by surveillance and described mostly in older (> 8 years of age) asymptomatic or fallen cattle (Konold et al., 2012). While the origin of C-BSE is attributed to consumption of contaminated foodstuffs, atypical BSE presentations have been suggested to represent sporadic or genetic prion diseases of cattle (Dudas et al., 2010).

1.4.3. Transmissible mink encephalopathy emerged during 1947 in ranch-raised mink (*Mustela vision*) in Brown County, Wisconsin, USA. During this outbreak, mortality reached 100% in mink over one year of age. Several pregnant females transferred to another farm developed TME while none of their born kits developed the disease (Hartsough and Burger, 1965). These observations suggest that exposure to the pathogenic agent occurred before transfer to the new farm and maternal transmission is not involved in the dissemination of TME.

With incubation periods of at least seven months, disease onset is subtle with changes in behaviour and cleanliness, including unkempt nests and pens. TME-affected mink present with difficulty eating and swallowing and often become excitable. As the disease progresses, affected mink arch the tail, develop motor incoordination and display convulsions. Somnolence and emaciation precede death. The clinical phase can last between 2 and 6 weeks (Hartsough and Burger, 1965; Marsh et al., 1991). Variation in clinical manifestation occurred in experimentally infected mink (Marsh, Burger and Hanson, 1969). As described by Marsh and coworkers, some mink became aggressive and others were shy and afraid when approached (Marsh, Burger and

Hanson, 1969). Also, while some mink would circle as if chasing their tails, for others disease onset was characterized by anorexia only.

Epidemiological investigations on the 1947 outbreaks and subsequent episodes during 1961 and 1963 pointed to the use of contaminated feedstuffs as the cause of the TME. Experimental transmission studies in mink supported this hypothesis (Burger and Hartsough, 1965). Unlike TME-free farms, the feed in those with affected mink was supplemented with cattle (meat from downer cattle), sheep, horse and other animal by-products. Although usage of sheep by-products in the diet of affected mink was rarely confirmed (Marsh and Hanson, 1979), the characteristics of the disease pointed to scrapie as the origin of TME. Marsh and Hanson (1979) exposed mink to scrapie isolates from the United Kingdom (UK) and United State (US). Only the mink intracranially or intramuscularly inoculated with the US isolates developed neurological disease with incubation periods distinct of TME. Also, none of the isolates produced disease following oral exposure suggesting that consumption of scrapie-contaminated meals did not cause TME.

The last recorded TME outbreak occurred in 1985 on a farm in Stensonville, Wisconsin. Epidemiological investigations and transmission studies re-focused the attention on the usage of downer cattle as the source of infection (Marsh et al., 1991). Experimental oral exposure of mink to BSE isolates resulted in neurological disease with incubation periods ranging between 14 and 16 months (Robinson et al., 1994). Despite confirmation of mink infection with a natural TSE, the disease presentation and neuropathology were different from those of mink with TME. These results support the hypothesis that an unrecognized spongiform encephalopathy of cattle was the

source of TME (Marsh and Bessen, 1993). Comparative transmission studies suggest a link between atypical BSE and TME outbreaks (Baron et al., 2007; Comoy et al., 2013).

1.4.4. Chronic wasting disease is contagious among cervids. CWD was first identified in captive mule deer (*Odocoileus hemionus*) from Colorado in 1967, and it was formally diagnosed as a TSE in 1978 (Williams and Miller, 2002; Williams, 2005). CWD has since spread into wild mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), elk (*Cervus canadensis*) and moose (*Alces alces*) from several regions of North America (Figure 1.2). CWD has also been detected in South Korea and Norway cervids.

The origin of CWD and the precise time and place of emergence remains unknown. Epidemiological data suggests the most likely sites of appearance in Colorado and Wyoming (Miller et al., 2000). The first described cases occurred in research facilities of the above-mentioned areas. These herds consisted of captured cervids from different wild populations, including pregnant females that were released after parturition. Transfer of animals between facilities was a standard practice (Williams and Young, 1980). CWD subsequently occurred in elk from these establishments (Williams and Young, 1980). In the following decades since first described, CWD appeared in free-ranging elk, mule deer, white-tailed deer and moose (Spraker et al., 1997; Williams and Miller. 2002; Baeten et al., 2007).

Cervid migrations and commercial movement of preclinical infected animals contributed to the geographic expansion of CWD into free-ranging and captive populations (Miller et al., 2000; Williams and Miller, 2002). To date, CWD occurs in at least 24 U.S. states (Colorado, Illinois, Iowa, Kansas, Maryland, Michigan, Minnesota, Missouri, Montana, Nebraska, New Mexico, New York, North Dakota, Ohio, Oklahoma, Pennsylvania, South Dakota, Texas, Utah, Virginia, West Virginia, Wisconsin, and Wyoming) and two Canadian provinces (Saskatchewan and Alberta) (Figure 1.2).

Figure 1.2. Geographic distribution of captive and wild cervid populations affected by Chronic Wasting Disease in North America. Taken from National Wildlife Health Center. U.S Geological Survey (https://www.nwhc.usgs.gov/disease_information/chronic_wasting_disease/).

Distribution of Chronic Wasting Disease in North America

CWD in free-ranging populations

Known distribution prior to 2000 (free-ranging)

CWD in captive facilities (depopulated)

CWD in captive facilities (current)

All locations are approximations based on best-available information
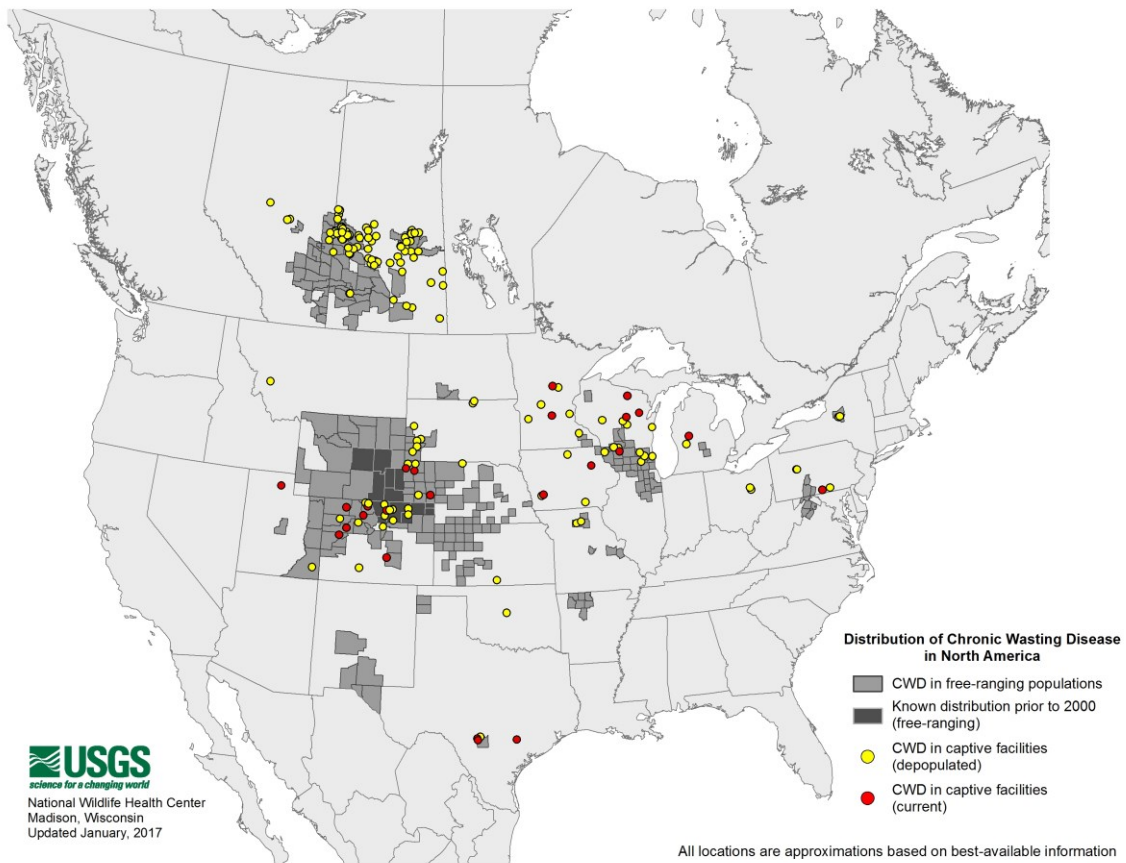
USGS
science for a changing world

National Wildlife Health Center
Madison, Wisconsin
Updated January, 2017

CWD appeared in farmed elk of Canada in 1996. Epidemiological studies suggested the infection was introduced into Saskatchewan farms by importing captive elk from a farm in South Dakota (Williams and Miller., 2002). The origin of the CWD epidemic in Canada's wild cervids remains unknown. Transmission by contact exposure between wild deer and infected farmed elk is a possibility (Kahn et al., 2004).

CWD outbreaks in captive cervids from farms in South Korea occurred by cohabitation with asymptomatic infected elk and deer imported between 1994 and 2003 from a farm in Saskatchewan, Canada. That farm was later determined to contain CWD-infected animals (Kim et al., 2005). A direct consequence of these outbreaks was the transmission into South Korean captive red deer (*Cervus elaphus*), sika deer (*Cervus Nippon sp.*) and crosses of these two species (commented in Lee et al., 2013b). In South Korea there is also confirmed transmission into other species, including a captive reindeer (*Rangifer sp*), Manchurian sika deer (*Cervus sp*) and one wild Korean water deer (*Hydropotes inermis*) (Prion 2016 conference communication).

Recently CWD was identified in wild reindeer (*Rangifer tarandus tarandus*) and moose from Norway, suggesting CWD is emerging in Europe's cervids (Benestad et al., 2016). The infectious origin or etiology associated with these cases and the current prevalence of CWD in Norway is unknown (Benestad et al., 2016). No record of CWD cases exists in the wild North American subspecies of caribou (*Rangifer tarandus sp*).

The incubation period of CWD in wild cervids is unknown. The youngest identified CWD-affected wild-ranging deer and elk indicated minimum incubation times of 16 and 21 months, respectively (Williams and Miller, 2002; Spraker et al., 1997). An age range of 2.5 to 7.5 years has been reported for free ranging CWD-affected deer and 1.8 to 10.5 years for elk (Spraker et al., 1997). Outbreaks in captive cervids occur in animals of various ages, ranging from 2 to 7.2 years for deer and 2.9 to 8.1 years for elk (Miller, Wild and Williams, 1998; Miller and Wild, 2004). Older animals have developed CWD in captivity, including 15 and 12-year-old elk and mule deer, respectively (Williams, 2005).

The clinical progression and symptomatology of CWD has been described in captive or experimentally infected cervids and can vary within and between species (Williams and Young, 1980; Fox et al., 2006; Johnson et al., 2011). Most prominent clinical features include behavioural alterations and progressive deterioration of body condition (i.e. weight loss). Following disease onset, these signs worsen over the course of weeks to months (Williams, 2005). Affected cervids may modify their interactions with herd mates or handlers and may display repetitive movement, periods of somnolence, fixed stares and hyperexcitability when approached. Altered postures with lowered head and ears, arching of the back and ataxia can be displayed (Williams and Miller, 2002; Fox et al., 2006; Johnson et al., 2011). The advanced clinical disease may involve odontoprisis, polydipsia, polyuria, difficulty swallowing, regurgitation of rumen contents and excessive salivation with drooling. Following recumbency, aspiration pneumonia, dehydration or hypothermia during the winter season are the most likely causes of death (Williams and Miller, 2002). Compared to deer, elk with CWD can present with nervousness and hyperesthesia and are

more likely to display motor disturbances and less commonly to develop polydipsia (Williams, 2005).

The primary mode of transmission between cervids is thought to be by direct animal-to-animal interactions (Miller et al., 2000; Miller and Williams. 2003). Experimental infection in pregnant muntjac deer (*Muntiacus reeves*) and detection of PrP^{CWD} in tissues from wild pregnant elk dams provide evidence for *in utero* transmission (Nalls et al., 2013; Selariu et al., 2015). In mule deer, however, maternal transmission has been shown to play a minor, or no, role compared to lateral transmission (Miller and Williams, 2003). CWD can also spread indirectly, for example by exposure of naïve cervids to CWD contaminated habitats (Miller and Williams, 2004, Mathiason et al., 2009; Moore et al., 2016). Affected cervids release CWD infectivity into the environment through secretions (saliva), excretions (urine and feces) and decomposing carcasses (Mathiason et al., 2009; Haley et al., 2011). Rapid pre-clinical accumulation of PrP^{CWD} aggregates in lymph tissues associated with the alimentary and intestinal mucosa suggests that CWD prions are most likely acquired via the oral route (Sigurdson et al., 1999; 2002; Fox et al., 2006). Prions have been demonstrated to bind soil microparticles while remaining infectious (Johnson et al., 2006b). Inhalation of CWD aerosols has also been proposed as a mechanism of exposure (Denkers et al., 2013). In addition to this, CWD infectivity is present in antlers. Given that males fight one another during the mating season, intradermal inoculation represents another potential route of exposure (Angers et al., 2009).

The overlapping of cervid habitats with agricultural animals raises concern for the susceptibility of livestock to CWD infection. Transmission studies using intracranial inoculation of CWD into cattle and sheep resulted in disease transmission, demonstrating that these species are susceptible to CWD prions (Hamir et al., 2005; Hamir et al., 2006a; Hamir et al., 2007). Considering the zoonotic potential of prions, as exemplified by the emergence of vCJD at the end of the BSE epidemic, the susceptibility of humans to CWD is of importance. Experimental transmission in non-human primates suggests humans have some degree of resistance to CWD infection (Race et al., 2014).

1.5 Prion diseases of humans

1.5.1. <u>Kuru</u> is an eradicated prion disease that reached epidemic proportions in the *Fore* people and surrounding linguistic groups from the highlands of Papua New Guinea in the late 1950's (Gajdusek and Zigas, 1957). Because of the disease presentation, affected groups named the disease kuru, which in *Fore* means "to be afraid" and "to shiver or shake". The clinical course involves progressive ataxia and tremors in the trunk, the head, and extremities. Advanced clinical signs include incontinence and speech loss. The clinical phase rarely lasted more than a year. Following disease onset, most cases succumbed within three to six months (Gajdusek and Zigas, 1957). Traditional oral history suggests the first cases appeared in the 1920s (Collinge et al., 2008; Collinge, 2008). Medical investigations began in 1955, and by 1959, kuru was more prevalent in adult females (60% of cases), adolescents and children (similar rates in both sexes). During this period, only 2% of the cases were adult males (Alpers, 2008). The age of onset ranged between 4 years and 70 years (Alpers, 2008). Neuropathological similarities highlighted by William Hadlow

between kuru and scrapie, encouraged experiments to evaluate the transmissible nature of kuru (Hadlow, 1995). When Gajdusek, Gibbs, and Alpers (1966) achieved transmission to chimpanzees, it became clear that traditional mortuary endocannibalism was spreading the disease. This practice was stopped in 1957 and was followed by a progressive decline and eradication of the epidemic (Collinge et al., 2006). Kuru cases born before the stoppage of cannibalism and identified between 1996 and 2004 suggest incubation periods can exceed 50 years (Collinge et al., 2006; Collinge et al., 2008). Based on transmission experiments in transgenic and normal mice it was proposed that kuru spread into the *Fore* as result of consumption of tissues from a sporadic Creuztfeld-Jakob disease case (Wadsworth et al., 2008; Asante et al., 2015).

1.5.2. Sporadic Creutzfeldt-Jakob disease (sCJD) is a rare neurodegenerative disease of undefined cause that accounts for approximately 80% of human prion disease cases. The incidence is approximately one case per million people. This form of CJD occurs predominantly in people between 45 to 75 years old with an average age of onset of 65 years (Parchi et al., 1999). Variations in clinical and histopathological presentations of sCJD are recognized and can be divided into those with a predominant cognitive decline and those with motor dysfunction (Parchi et al., 1999). The sCJD presentations involve differences in the average age and clinical signs at onset, disease duration, clinical features during progression and electroencephalogram readings (Parchi et al., 1999). Cases of sCJD are routinely categorised based on the clinical signs, histopathology, PK-res PrP^{Sc} profile on SDS-PAGE (type 1 or 2) and the patient's PrP^{C} sequence at amino acid 129(M/V) (Parchi et al., 1997; Parchi et al., 1999). This classification results in six sCJD subtypes (MM1, MV1, MM2, VV1, VV2 and MV2). According to their transmission

properties in transgenic mice, these subtypes can be further grouped (MM1 and MV1), (MV2 and VV2), (MM2) and (VV1) (Bishop, Will and Manson, 2010). These groups were consistent with previous observations following transmission of a large cohort of CJD cases into primates (Parchi et al., 1999).

1.5.3. Iatrogenic Creutzfeldt-Jakob disease (iCJD) is an acquired prion disease that resulted from inadvertent medical and therapeutic exposure of people to CJD infectivity. This mode of transmission became evident after the recipient of a corneal transplant developed CJD 18 months after the surgery. The donor was later confirmed to have died of CJD (Duffy et al., 1974). Two more cases occurred in patients that were evaluated by stereotactic electroencephalogram. The apparently sterile electrodes were previously implanted in a patient with confirmed CJD (reviewed in Brown et al., 2012). Implantation of a suspected electrode in the cortex of a chimpanzee resulted in spongiform encephalopathy, confirming that sterilization procedures were not sufficient to decontaminate CJD infectivity (reviewed in Brown et al., 2012). Iatrogenic transmission of CJD has also occurred through injection of human derived growth hormones or use of dura mater grafts obtained from human cadavers (reviewed in Brown et al., 2012). Improved CJD diagnostics, decontamination procedures and use of recombinant hormones have contributed to minimizing the risk of iatrogenic transmission (Rudge et al., 2015). Secondary transmission of variant CJD (vCJD) through blood transfusion has resulted in various cases. Epidemiological investigations have revealed these persons belonged to a group of 67 people that receive blood from donors that succumbed to vCJD (Urwin et al., 2015).

1.5.4. Variant Creutzfeldt-Jakob disease (vCJD) is a form of acquired CJD (Will et al., 1996). The emergence of this new form of CJD during the decline of BSE in the UK led Will and coworkers (1996) to suggest vCJD resulted from ingestion of BSE-contaminated foodstuffs. This hypothesis is supported by transmission and neuropathological studies in rodent models (Bruce et al., 1997; Hill et al., 1997). These studies revealed the BSE and vCJD agents have similar transmission properties and produce similar brain lesion profiles (Bruce et al., 1997; Hill et al., 1997). A total of 179 UK cases and 53 cases distributed among 11 other countries have been reported since vCJD was first described (Maheshwari et al., 2015). The age of onset ranges from 12 to 74 years, with the average being 28 years. The clinical course can last between 6.5 to 40 months. A large proportion of cases developed non-specific psychiatric features (i.e. depression, anxiety, and apathy) in the early stages while others were characterized by neurological signs like abnormal or painful sensory symptoms (Will et al., 1996; Heath et al., 2011). Cognitive dysfunction, ataxia, involuntary movements and progressive dementia can occur as the disease worsens (Will et al., 1996). Electroencephalogram results do not resemble those of sCJD (Heath et al., 2011). Compared to sCJD, the most significant neuropathological feature of vCJD is the abundant accumulation of PrP^{Sc} plaques in the cerebrum and cerebellum (Will et al., 1996). As will be described in more detail later, there is strong genetic linkage with the PrP^{C} polymorphism at amino acid 129 (M/V).

1.5.5. Genetic prion diseases in humans

Approximately 15% of all prion disease cases occur in people carrying autosomal dominant *PRNP* mutations that affect the structure of PrP^{C}, making it more prone to misfold and aggregate. These

modifications can involve single amino acid changes, the introduction of premature stop codons and sequence duplications (Mastrianni, 2010; Kaski et al., 2011). The most common genetic forms of prion disease include familial Creutzfeldt-Jakob disease (fCJD), Gerstmann-Sträussler-Scheinker (GSS) and fatal familial insomnia (FFI). Several *PRNP* mutations increase the risk of developing each of these inherited forms of prion disease (Mastrianni, 2010; Lloyd, Mead and Collinge, 2013). These mutations can also result in different disease phenotypes. The onset of clinical disease, the disease duration, symptomatology and neuropathology relate to the particular mutation a person carries. Also, the prion disease phenotype can vary within the same carrier families (Mastrianni, 2010; Lloyd, Mead and Collinge, 2013). The presence of the 129(M/V) polymorphisms in the mutated PrP has been shown to modify the disease penetrance and the clinical presentation (Mastrianni, 2010; Kaski et al., 2011).

1.6 The prion protein

The cellular prion protein (PrP^C) is a membrane glycoprotein encoded by a highly conserved mammalian gene (i.e. *PRNP* or *Prnp*). PrP^C is predominantly expressed in nervous tissues, but can also be found in non-neural cells (i.e. lymphoid tissues) (Oesch et al., 1985; Kretzschmar et al., 1986; Ford et al., 2002).

The amino acid composition of PrP^C varies depending on the species; the immature PrP^C polypeptide ranges between 253 to 264 amino acids in length. Before undergoing normal post-translational processing, the cervid PrP^C is composed of 256 amino acids (Figure. 1.3).

Figure 1.3. Sequence alignment of the PrP^C primary structures of the most commonly affected cervid species. The primary structures include the endoplasmic reticulum translocation signal (N-terminus; amino acid 1 to 24) and the GPI attachment signal (C-terminus; amino acid 235 to 256) (Stahl et al., 1987; Turk et al., 1988). Boxes delimit species-specific amino acid polymorphisms. The effect of these polymorphisms will be described in sections covering genetic susceptibility.

```
        10          20          30          40          50
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Odocoileus virginianus wt
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Odocoileus virginianus S96
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Odocoileus virginianus H95
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Odocoileus hemionus F225
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Odocoileus hemionus wt
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Cervus canadensis L132
MV K S H I  G S W I  L V L F  V A M W S D V G L  C K K R P K P  G G G W N T  G G S  R Y P  G Q G S  P G G N R Y    Cervus canadensis M132

        60          70          80          90          100
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G Q G G T  H S Q W N K    Odocoileus virginianus wt
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G Q[S]G T  H S Q W N K    Odocoileus virginianus S96
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G[H]G G T  H S Q W N K    Odocoileus virginianus H95
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G Q G G T  H S Q W N K    Odocoileus hemionus F225
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G Q G G T  H S Q W N K    Odocoileus hemionus wt
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G Q G G T  H S Q W N K    Cervus canadensis L132
P P Q G G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G  W G Q P  H G G G G  W G Q G G T  H S Q W N K    Cervus canadensis M132

        110         120         130         140         150
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y M L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Odocoileus virginianus wt
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y M L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Odocoileus virginianus S96
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y M L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Odocoileus virginianus H95
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y M L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Odocoileus hemionus F225
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y M L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Odocoileus hemionus wt
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y[L]L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Cervus canadensis L132
P S K P  K T N M K  H V A G A A A A G A V V G G L  G G Y M L  G S A M S  R P L I  H F  G N D Y E  D R Y Y R E N    Cervus canadensis M132

        160         170         180         190         200
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Odocoileus virginianus wt
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Odocoileus virginianus S96
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Odocoileus virginianus H95
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Odocoileus hemionus F225
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Odocoileus hemionus wt
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Cervus canadensis L132
M Y R Y P  N Q V Y  Y R P  V D Q Y N N Q N T F  V H D C V N I  T V K Q H T V T T T T K G E N F  T E T D I  K M    Cervus canadensis M132

210         220         230         240         250
M E R V V E  Q M C I  T Q Y Q R E S  Q A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Odocoileus virginianus wt
M E R V V E  Q M C I  T Q Y Q R E S  Q A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Odocoileus virginianus S96
M E R V V E  Q M C I  T Q Y Q R E S  Q A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Odocoileus virginianus H95
M E R V V E  Q M C I  T Q Y Q R E[F]Q A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Odocoileus hemionus F225
M E R V V E  Q M C I  T Q Y Q R E S  Q A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Odocoileus hemionus wt
M E R V V E  Q M C I  T Q Y Q R E S[E]A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Cervus canadensis L132
M E R V V E  Q M C I  T Q Y Q R E S[E]A Y Y Q R G A S  V I L F S S P P V I  L L I  S F L I  F L I  V G    Cervus canadensis M132
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During synthesis, the signal peptides in the N-terminus and C-terminus target the nascent PrP^C polypeptide to the endoplasmic reticulum (ER) and favour its attachment to the ER membrane (Stahl et al., 1987; Turk et al., 1988). In cervids, the first 24 N-terminal amino acids comprise the endoplasmic reticulum translocation signal. The last 23 C-terminal residues prompt the attachment of a glycosyl-phosphatidylinositol (GPI) anchor that secures PrP^C to the exoplasmic face of the ER and subsequently to the cell surface (Stahl et al., 1987; Turk et al., 1988). Both of these signal peptides are cleaved from PrP^C during its journey to and through the ER yielding cervid PrP^C molecules composed of 209 amino acids (Figure 1.4A).

As PrP^C adopts its three-dimensional structure during its ER transit, the formation of a disulfide bond occurs between cysteines 179 and 214 (Cys182 and Cys217 in the cervid protein) (Turk et al., 1988). On its way to the cell membrane, during the traffic between the ER and Golgi, PrP^C undergoes asparagine (N)-linked glycosylation on residues 181 and 197 (N184 and N200 in the cervid protein) (Haraguchi et al., 1989). PK treatment of brain homogenates containing PrP^{Sc} results in three PK-res PrP^{Sc} glycoforms resolved by SDS-PAGE (di-glycosylated, mono-glycosylated and unglycosylated).

Figure 1.4. Linear representation of the cervid cellular prion protein. A) Full length, membrane-anchored PrP^C after removal of the signal peptides, formation of the disulfide bond (S-S) and glycosylation (CHO). B) C2-PrP^C after experiencing β-cleavage near the octarepeat (OR) region. C) C1-PrP^C after experiencing α-cleavages near and within the hydrophobic domain (HD). The globular domain is composed by the β-sheet (β1), α-helix (α1), β-sheet (β2) α-helix 2 (α2) and α-helix (α3).
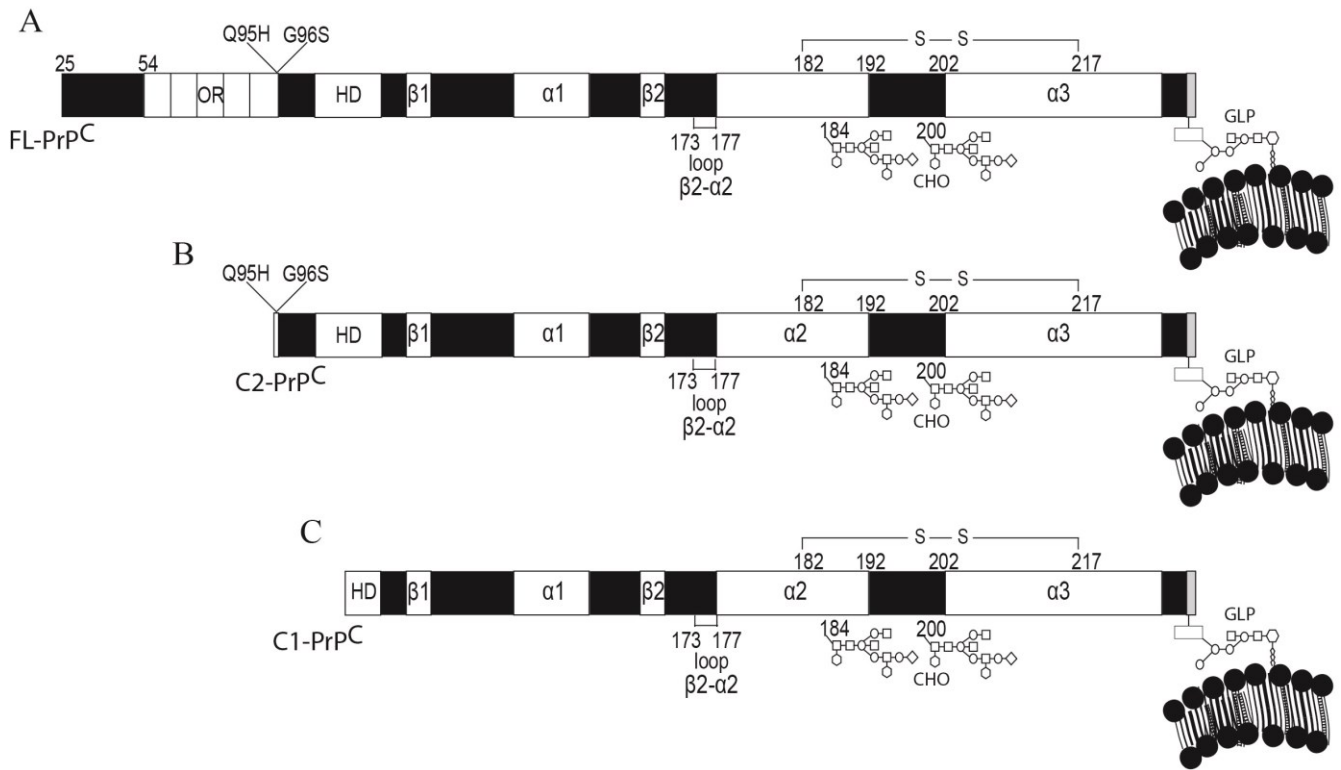
Structurally mature PrP^C consists of a flexible (disordered) N-terminal domain, followed by the hydrophobic domain and the C-terminal globular domain (Riek et al., 1996; Zahn et al., 2000). The N-terminus of cervid PrP^C is composed of a 29 amino acid segment followed by five octapeptide repeats (OR) in most species (6 in cattle and squirrel monkeys). These peptide repetitions are rich in proline (P), glycine (G), glutamine (Q), histidine (H) and tryptophan (W) and been shown to bind Cu^{2+}/Zn^{2+} (reviewed in McDonald and Millhauser, 2014). Duplications of these repeats have been associated with genetic prion diseases highlighting the relevance of this PrP^C domain (Mastrianni, 2010). Binding of metal ions through the OR has been proposed to represent one of the many, and still under debate, putative functions of PrP^C (reviewed in Lau et al., 2015). In cell culture studies, addition of copper and zinc resulted in reversible endocytosis of PrP^C suggesting a role in uptake and trafficking of these metal ions (Pauly and Harris, 1998; Brown and Harris, 2003). Metal binding has been shown to occur through the histidine residues in the HGGGW segments of 4 octarepeats (Chattopadhyay et al., 2005). The amount of copper bound has structural and functional implications in the OR. Depending on the level of copper occupancy, the N-terminus of PrP^C can adopt one of three different structural arrangements (Chattopadhyay et al., 2005). Lau and co-workers (2015) identified various functional effects in transgenic mice expressing locked states of each of these OR structures. Compared to mice with other OR arrangements, those expressing the one with the lowest level of copper occupancy resulted in shorter incubation periods and different clinical presentation following prion infection (Lau et al., 2015). Low copper occupancy was also associated with differences in N-terminal endoproteolysis of PrP^C demonstrating the importance of this domain during health and disease (Lau et al., 2015).

The N-terminus of PrP^C can undergo various cleavages resulting in C-terminal fragments that can be differentiated based on antibody reactivity and migration in SDS-polyacrylamide gels (Figure 1.4B). One of these cleavages (β-cleavage) occurs at the edge of the OR producing a fragment with a reported molecular weight of approximately 20 kDa (termed C2-PrP) (Chen et al., 1995; Yadavalli et a., 2004).

Increased β-cleavage products have been reported in brains of CJD patients and scrapie-infected mice compared to brains of normal healthy controls (Chen et al., 1995; Yadavalli et a., 2004). Unlike the β-cleavage products found in controls (C2-PrP^C), disease-specific C2-PrP (C2-PrP^{Sc}) is protease resistant and insoluble in detergents. Based on these and other properties that include migration in SDS polyacrylamide gels and the presence of N-terminal epitopes, C2-PrP^{Sc} is thought to be an "*in vivo* homolog" of PrP27-30 (Chen et al., 1995). Although the mechanistic origin of C2-PrP^{Sc} and its role in pathogenesis remain to be clarified, differences in N-terminal endoproteolysis have been observed upon prion transmission between different species (Jeffrey et al., 2006a; Jeffrey et al., 2006b).

Another N-terminal cleavage (α-cleavage) occurring between Lys109 and His100 (Lys113 and 114His in the cervid protein) results in a PrP^C fragment (C1-PrP^C) with an apparent molecular weight of 16 kDa (Figure 1.4C). Other α-cleavages (α2 and α3) occur between Ala116-Ala117 and Ala119-Val120 (reviewed in McDonald and Millhauser, 2014). Unlike C2-PrP^{Sc} fragments, C1-PrP^C is soluble in detergents and sensitive to proteolysis (Chen et al., 1995; Yadavalli et a., 2004).

The α-cleavage of PrP^C has been proposed to exert a protective effect during prion infection (Westergard et al., 2011). This effect is thought to occur through the proteolytic removal of amino acids required for the stabilizing the core of PrP^{Sc} (Hölscher et al., 1998). Peptide sequencing analysis and detection with epitope-specific antibodies demonstrate that PrP27-30 and C2-PrP^{Sc} do not undergo α-cleavages (Oesch et al., 1985; Chen et al., 1995). The α-cleavages take place near or within a region of PrP^C that is rich in hydrophobic amino acids, referred to as the hydrophobic domain (HD). The HD is of particular importance for the formation of PrP^{Sc} (Hsiao et al., 1990; Hölscher et al., 1998).

The majority of mutations predisposing for familial prion diseases appear to destabilize the C-terminus of PrP^C (Mastrianni, 2010). This domain of PrP^C is known to adopt a three-dimensional structure composed by a tandem of secondary structures. These include one β-sheet (β1) followed by an α-helix (α1) and a second β-sheet (β2) followed by two more α-helices (α2 and α3)(Riek et al., 1996; Zahn et al., 2000; Baral et al., 2015). The segment connecting the β-sheet 2 and α-helix 2 (β2-α2 loop) has drawn attention since the structure of PrP^C was determined for different species (Riek et al., 1996; Zahn et al., 2000; Sigurdson et al., 2010; Baral et al., 2015). The amino acid sequence of the β2-α2 loop has been shown to differ between species (Figure 1.5). This amino acid variability is associated with flexible (SNQNN) and rigid (NNQNT) topologies that have been hypothesized to control prion transmission between species (Riek et al., 1996; Raymond et al., 2007; Kurt et al., 2014). The exact structure of PrP^{Sc} remains unresolved and various models have been proposed that are under dispute, however, the secondary structures of PrP^C are known to be absent in PrP^{Sc}, which is mainly composed of β-sheet arrangements (Vazquez-Fernandez et al., 2016).

Figure 1.5. Sequence alignment from mammalian species with different PrP^C β2-α2 loops. Species expressing SNQNN produce loops with flexible topology and are present in diverse species including humans and mice. Cervids are characterized by rigid loop structures (NNQNT). The sequences are presented according to the human amino acid numbering. Boxes delimit amino acid differences.

```
                    10            20           30            40           50
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPP   Odocoileus virginianus wt
--MANLGYWLLALFVTMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP   Mus musculus
--MANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP   Homo sapiens
--MANLSYWLLALFVATWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP   Mesocricetus auratus
--MANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP   Macaca fascicularis
--MANLGCWMLVLFVATWSDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP   Saimiri sciureus
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPP   Cervus canadensis M132


                               60           70           80            90
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-THS   Odocoileus virginianus wt
QGG--------TWGQPHGGGWGQPHGGSWGQPHGGSWGQPHGGG-WGQGGGTHN   Mus musculus
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGTHS   Homo sapiens
QGG-------GTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGTHN   Mesocricetus auratus
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGTHN   Macaca fascicularis
QGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGTHN   Saimiri sciureus
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-THS   Cervus canadensis M132


    100          110          120          130          140          150
QWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR   Odocoileus virginianus wt
QWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPMIHFGNDWEDRYYR   Mus musculus
QWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYVLGSAMSRPIIHFGSDYEDRYYR   Homo sapiens
QWNKPNKPKTSMKHMAGAAAAGAVVGGLGGYMLGSAMSRPMLHFGNDWEDRYYR   Mesocricetus auratus
QWHKPSKPKTSMKHMAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR   Macaca fascicularis
QWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR   Saimiri sciureus
QWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR   Cervus canadensis M132


    160          170          180          190          200
ENMYRYPNQVYYRPVDQY|NNQNT|FVHDCVNITVKQHTVTTTTKGENFTETDIKM   Odocoileus virginianus wt
ENMYRYPNQVYYRPVDQY|SNQNN|FVHDCVNITIKQHTVTTTTKGENFTETDVKM   Mus musculus
ENMHRYPNQVYYRPMDEY|SNQNN|FVHDCVNITIKQHTVTTTTKGENFTETDVKM   Homo sapiens
ENMNRYPNQVYYRPVDQY|NNQNN|FVHDCVNITIKQHTVTTTTKGENFTETDVKM   Mesocricetus auratus
ENMYRYPNQVYYRPVDQY|SNQNN|FVHDCVNITIKQHTVTTTTKGENFTETDVKM   Macaca fascicularis
ENMYRYPSQVYYKPVDQY|SNQNN|FVHDCVNVTIKQHTVTTTTKGENFTETDVKM   Saimiri sciureus
ENMYRYPNQVYYRPVDQY|NNQNT|FVHDCVNITVKQHTVTTTTKGENFTETDIKM   Cervus canadensis M132
                   β2-α2

    210         220          230          240          250
MERVVEQMCITQYQRESQAYYQ--RGASVILFSSPPVILLISFLIFLIVG   Odocoileus virginianus wt
MERVVEQMCVTQYQKESQAYYDGRRSSSTVLFSSPPVILLISFLIFLIVG   Mus musculus
MERVVEQMCITQYERESQAYYK--RGSSMVLFSSPPVILLISFLIFLIVG   Homo sapiens
MERVVEQMCVTQYQKESQAYYDGRRSS-AVLFSSPPVILLISFLIFLIVG   Mesocricetus auratus
MERVVEQMCITQYEKESQAYYQ--RGSSMVLFSSPPVILLISFLIFLIVG   Macaca fascicularis
MERVVEQMCITQYEKESQAYYQ--RGSSMVLFSSPPVILLISFLIFL      Saimiri sciureus
MERVVEQMCITQYQRESEAYYQ--RGASVILFSSPPVILLISFLIFLIVG   Cervus canadensis M132
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1.7 Prion replication

PrP^C and PrP^{Sc} have different structural and operational properties (i.e. resistance to proteolysis and solubility in detergents). The mechanism by which prions replicate remains a matter of debate; however, it is thought to involve the conformational conversion of PrP^C into PrP^{Sc}. Two theoretical models have been proposed to explain this process:

The template-directed refolding model states that upon spontaneous misfolding of PrP^C into PrP^{Sc} (sporadic disease) or following exposure to infectious material, one PrP^{Sc} monomer undergoes a structural interaction with a PrP^C monomer through the formation of a heterodimer. After the energy barrier required to rearrange the globular domain of PrP^C is diminished, conversion into PrP^{Sc} occurs. The resulting PrP^{Sc} homodimer would dissociate, and the process would be repeated with additional PrP^C monomers (Prusiner et al., 1990).

An alternative hypothesis, referred as the seeded nucleation polymerization model, proposes that within a host, PrP^C and PrP^{Sc} exist in a reversible equilibrium that favors the structure of PrP^C, however, small amounts of PrP^{Sc} can exist. Formation of the seed or polymer would occur slowly, but once sufficient PrP^{Sc} mass accumulates rapid recruitment of PrP^C monomers would follow. Fragmentation of larger aggregates would generate more seeds that would increase the conversion of additional PrP^C molecules (Jarrett and Lansbury, 1993).

1.8 Pathogenesis

Neuropathology of prion diseases involves spongiform degeneration, astrogliosis and accumulation of transmissible PrP^{Sc} aggregates (Figure 1.1). It remains unclear how PrP^{Sc} induces the progressive neurodegeneration and brain pathology observed in patients and animals.

Expression of PrP^C is necessary for PrP^{Sc} replication and production of disease (Bueler et al., 1993). Prion infection in *Prnp* knockout mice that previously received brain grafts from mice overexpressing PrP^C resulted in neuropathology only in the grafted areas, further implicating PrP^C in the process (Brandner et al., 1996). This study also showed that despite migration of PrP^{Sc} from the graft to the continuous PrP^C-deficient tissue no pathology developed.

A number of studies have shown that accumulation of PrP^{Sc} aggregates and infectivity is not directly neurotoxic (Hill et al., 2000; Race et al., 2002a; Bian et al., 2017). Various interspecies transmission studies have shown that certain rodent models can survive despite accumulation of significant levels of infectivity and PrP^{Sc} deposits (Hill et al., 2000; Race et al., 2002a; Bian et al., 2017). Even after second passage of PK-res positive brain homogenates from donors that expressed the same PrP^C amino acid sequence as the recipient host, some mice failed to develop the disease (Race et al., 2002a; Bian et al., 2017). The study by Bian et al (2017) is quite intriguing; clinically affected mice that accumulated PK-res PrP^{Sc} and infectivity after first passage, failed to transmit disease after second passage in recipient mice expressing homologous PrP^C molecules.

A hypothetical toxic PrP molecule (termed PrP^L) has been proposed to deliver the neurotoxicity effect during prion infection. This toxic product forms after PrP^{Sc} accumulation has overcome the host clearance (i.e. protein degradation) of abnormal PrP^{Sc} species (Sandberg et al., 2014). The existence of prion clearance mechanisms in the brain is evident from studies monitoring levels of infectivity after intracranial inoculation, identifying a decline of infectivity during the first days post inoculation (Bueler et al., 1993). Transgenic mice in which PrP^C expression could be down-regulated by oral administration of doxycycline 98 or 126 days after inoculation, resulted in a significant extension of the incubation period (Safar et al., 2005). Alternatively, it has been

proposed that PrP^{Sc} exerts its neurotoxicity through PrP^C (Herrmann et al., 2015). Modified antibodies that recognize the globular domain of PrP^C can trigger toxic effects that mimic those of prion infection (Hermann et al., 2015). These effects diminish after treatment with antibodies recognizing the flexible N-terminus. This hypothesis states that an interaction between PrP^{Sc} and the globular domain of PrP^C can induce structural transitions in the flexible domain of PrP^C that cause neurotoxic effects (Hermann et al., 2015).

1.9 Prion strains

Different clinical and pathological phenotypes in most prion-affected mammals are associated with strains capable of stable propagation of their epigenetic biological information. Different strains have been identified that correlate with classical and atypical scrapie presentations in sheep and goats, classical and atypical forms of BSE in cattle, sporadic and variant CJD in humans, and CWD in cervids.

The "scratching" and "nervous" syndromes in scrapie-affected sheep were the first recognizable prion disease phenotypes that were shown to be reproducible upon passage in goats (Pattison and Millson, 1961). Transmission of field and experimental scrapie into rodents favored the recognition of several strains. Pioneering transmission work with a scrapie isolate (SSBP/1: Sheep Scrapie Brain Pool 1) derived from Cheviot sheep resulted in the identification of various strains (i.e. 22C, 22A, 22L, etc.) that remain useful reagents for the study of prion diseases (See Kimberlin et al., 1989 for a summary of the SSBP/1 passage history). These strains differ in the specific brain lesions, clinical signs and incubation periods they produced in groups of inoculated inbred mice.

Quantification of the degree and distribution of spongiform change in different brain regions proved to be a useful tool to distinguish different prion strains (Bruce et al., 1997). As mentioned previously, transmission of brain tissues from vCJD and BSE cases into inbred mice resulted in similar brain lesion profiles, suggesting a common origin for these prion strains.

The properties of PrP^{Sc} represent another tool to differentiate between prion strains. Using hamster and mouse-adapted scrapie strains, Kascsak and coworkers (1985) suggested that specific structural differences between the PrP^{Sc} molecules of these strains could represent basis for their distinct pathological phenotypes. The strains analyzed were characterized by differences in sedimentation and morphology of purified PrP^{Sc} aggregates, resistance to proteases and specific infectivity (Kascsak et al., 1985).

In addition to providing experimental support that insoluble proteins can produce distinct protein aggregates with differing biochemical characteristics, the study identified PK resistance as a mean of distinguishing prion strains. PK-res PrP^{Sc} from two hamster-adapted TME strains resolved into distinct migration patterns by SDS-PAGE (Bessen and Marsh, 1992a; 1992b). Named after their associated clinical presentation in hamsters, these strains, hyperactive (HY) and drowsy (DY) produced PK-res PrP^{Sc} molecules with different migration profiles. The distinct gel migration profile of the Dy PK-res PrP^{Sc} fragments is associated with the amino acids at which PK proteolysis occurs. While in the Hy PrP^{Sc} the cleavages were located towards the N-terminus (G74, G82 and G90) those of the Dy PrP^{Sc} were more C-terminal (G92, G98 and L101) yielding smaller PK-res PrP^{Sc} fragments (Bessen and Marsh, 1994). Dy PrP^{Sc} also differs in the relative sensitivity to protease digestion with Hy PrP^{Sc} tolerating PK treatments for as long as 48 hours while Dy PrP^{Sc} degrades after a few hours. These results supported the concept that the PrP^{Sc}

conformation (three-dimensional shape) encodes and propagates the epigenetic identity of prion strains.

Further analysis of Hy and Dy PrP^{Sc} has revealed differences in their seeding activity during cell-free protein misfolding amplification (PMCA). This technique uses the PrP^C from uninfected brain homogenates as a substrate to amplify PrP^{Sc} in problem samples (Saa et al., 2005). Consistent with the incubation period of these strains upon the passage in hamster, Hy PrP^{Sc} was more efficient in seeding the PMCA reactions than Dy PrP^{Sc} (Ayers et al., 2011). The electrophoretic profile of the newly generated PK-res PrP^{Sc} resembled that of brain-derived PrP^{Sc}, supporting the idea that prion conformation directs the misfolding of PrP^C molecules.

The structural or conformational stability of PrP^{Sc} molecules against guanidine hydrochloride (GdnHCl) and denaturing detergents (SDS) also provides another tool to distinguish prion strains. Hy PrP^{Sc} is more resistant to denaturation than Dy PrP^{Sc} (Ayers et al., 2011). Despite sharing the same PrP^{Sc} amino acid sequence, these two strains produce different prion disease phenotypes and structurally distinct PrP^{Sc} molecules.

Similar observations occur in sCJD cases. Analysis of representative MM1 and MM2 sCJD cases revealed PK-res PrP^{Sc} (type 1 and type 2) molecules with distinct structural properties (Safar et al., 2015). In addition, a fraction of the PrP^{Sc} in the brain of these patients was shown to be prone to protease degradation (PK-sen PrP^{Sc}). Using conformational dependent immunoassay (CDI), a methodology that exploits the differential epitope accessibility of PrP^C and PrP^{Sc} and their susceptibility to GdnHCl unfolding, the total amount of PrP^{Sc} (PK-res PrP^{Sc} + PK-sen PrP^{Sc}) is

measured in an ELISA-based format (Safar et al., 1998). MM1 and MM2 sCJD cases contained significantly different the amounts of PK-res PrP^{Sc} and PK-sen PrP^{Sc} (Safar et al., 2015). Haldiman and colleagues (2013) have previously described sCJD cases that contain both PK-res PrP^{Sc} type 1 and type 2 (classified as MM1+2 sCJD).

Finally, the most important property that differentiates between prion strains is the ability to infect specific hosts, defined as the host range. As previously mentioned, vCJD cases contain a prion strain that resembles the characteristics of the strain associated with classical BSE (C-BSE)(Bruce et al., 1997; Hill et al., 1997). Transmission of vCJD and sCJD samples of equivalent *PRNP* genotype into rodents resulted in differential transmission despite vCJD and sCJD tissues expressing the same PrP amino acid sequence. While vCJD prions generated disease in normal FVB mice, sCJD failed to transmit (Hill et al., 1997).

Cervid prion strains also differ in their host range; while certain strains of mule deer CWD are not transmissible to hamsters others transmit more efficiently (Bartz et al., 1998; Raymond et al., 2007). BSE strains have different proclivities to infect mice; RIII and VM mice inoculated with C-BSE succumb to prion disease, whereas those infected with L-type BSE survive the infection (Capobianco et al., 2007).

1.10 Susceptibility to prion disease

The susceptibility of mammals to prion disease is dependent upon the properties of the propagating prion strain and the host PrP^{C} primary structure (Dickinson et al., 1968a). Using two

different inbred lines of laboratory mice (RIII and VM), Dickinson and co-workers (1968a) identified a differential susceptibility of mice to infection with the sheep scrapie-derived ME7 strain. While the RIII mice succumbed to prion disease 160 days post inoculation, the incubation period for VM mice exceeded 250 days. Inoculation of ME7 in crosses of these mice lines resulted in intermediate incubation periods. Dickinson's pioneering work suggested that a single gene with two alleles s7 (short) and p7 (prolonged) were affecting the incubation period and designated the gene as *Sinc* (for scrapie incubation) (Dickinson et al., 1968a). Dickinson and Meikle (1971) then compared the incubation patterns produced by ME7 and 22A (another sheep scrapie derived strain) in s7 (C57BL) and p7 (VM) mice. On this occasion, inoculation of 22A in VM mice resulted in shorter incubation periods compared to the long incubation period of ME7. However, unlike ME7, infection of C57BL mice with the 22A strain produced incubation times larger than 400 days (Dickinson and Meikle, 1971). With the revelation that PrP27-30 results from misfolding of a host-encoded protein (PrP^C), the gene encoding it (*Prnp*) was linked to the *Sinc* gene (Oesch et al., 1985; Carlson et al., 1986). Two alleles (*Prnp^a and Prnp^b*) coding for amino acid polymorphisms 108 (L/F) and 189 (T/V) were associated with the incubation period differences (Westaway et al., 1987).

Dickinson and coworkers (1986b) also presented evidence for the existence of a similar gene controlling the incubation time of sheep exposed to scrapie. Selection experiments with various sheep breeds were established for and against the production of scrapie by the subcutaneous route. For the Cheviot selection line (Dickinson et al., 1968b), subcutaneous inoculation resulted in 37% of sheep developing scrapie with a mean incubation time of 313±9 days, however, after intracranial exposure, 59% came down with scrapie after mean incubation times ranging between

197±7 and 917±90 days post exposure. In subsequent experiments, transmission studies with the progeny of the "resistance" selection line resulted in no scrapie cases after subcutaneous inoculation. Exposure of sheep from the susceptible line to scrapie resulted in 80% of cases (Dickinson et al., 1968b). These results suggested that a single gene (Sip) with two alleles (sA and pA) controlled the resistance of sheep to subcutaneous infection with the SSBP1 isolate.

The *Sip* gene was later proved to be the *PRNP* gene (Goldmann et al., 1991). These studies revealed the PrP^C molecules in the susceptible and resistant Cheviot lines differed at amino acid positions 136 (V/A) and 171 (Q/R). Sheep that succumbed after subcutaneous inoculation with the SSBP/1 expressed $V136Q171-PrP^C$, while those from the resistant line produce $A136R171-PrP^C$ (Goldmann et al., 1991). Several amino acid polymorphisms occur in sheep; the potential amino acid combinations in the same PrP^C molecule or expression of two PrP^C molecules (heterozygosis) with different primary structure can reduce the chances of developing scrapie during the lifespan (Hunter et al., 1996). Sheep susceptibility to prion infection, as described for mice, also depends on the invading prion strain. A136R171-*PRNP* homozygous sheep are more susceptible to infection with BSE and other strains of scrapie (i.e. CH1641 and atypical) than are V136Q171 sheep (Hunter et al., 1996; Gotte et al., 2011).

M129-PRNP homozygous kuru patients were associated with earlier age of onset and shorter disease duration as compared with individuals expressing the *V129-PRNP* allele (Cervenáková et al., 1998). Subsequent comparison of the genotype frequencies in a sample of healthy and affected *Fore* people confirmed that both homozygous genotypes were susceptible. However, no homozygous 129M-*PRNP* women were found among the survivors (Lee et al., 2001). Genetic

analysis of *Fore* groups revealed that homozygosity for methionine was associated with high susceptibility, while low susceptibility groups contained an excess of heterozygotes and a deficit in methionine homozygotes (Mead et al., 2008). Also, *PRNP* genotypes in kuru patients with long incubation periods (>50 years) at the end of the epidemic supported the idea that heterozygosity (M129/V129 PrP^C allotype) confers resistance to *kuru* (Collinge et al., 2008). It was recently proposed that the polymorphism 127(G/V), which was under strong positive selection, could have prevented the *Fore* population collapse if cannibalism would not have stopped (Asante et al., 2015).

As previously mentioned, it has been proposed that emergence of vCJD in humans was caused by consumption of BSE prions (Will et al., 1996). This hypothesis is supported by comparative transmission studies (Bruce et al., 1997; Hill et al., 1997). Variation of amino acid M129V of the human PrP^C affects susceptibility to vCJD. The majority of vCJD cases have occurred in homozygous *129M-PRNP* patients while only two were heterozygous, suggesting the valine polymorphism is partially protective against BSE prions (Mok et al., 2017). Transmission studies in transgenic mice expressing either of these polymorphisms confirmed that 129V-PrP^C provides resistance to BSE prions (Wadsworth et al., 2004). A recent epidemiological study in archived appendix samples suggested that 1 in 2000 people in the UK are silent carriers of vCJD infectivity (Gill et al., 2013). The majority appendices that were positive for PrP^{Sc} were from people expressing the 129V polymorphism. These results indicate progression of vCJD is slower in people carrying this *PRNP* allele and future cases might occur.

Unlike susceptibility of humans to vCJD, iatrogenic Creutzfeldt-Jakob disease (iCJD) in UK patients treated with cadaveric growth hormone, manifested mainly in *129V-PRNP* homozygous and heterozygous individuals (reviewed by Brown et al., 2012). The *PRNP* genotypes of iCJD cases in other countries contrasted with those of UK cases; in France and the US, iCJD was reported predominantly in *129M-PRNP* homozygous and heterozygous persons (reviewed by Brown et al., 2012). Comparison of the 129-*PRNP* allelic frequencies in healthy and iCJD affected individuals from the UK and France indicates that growth hormones provided in both countries were contaminated with CJD agents of different properties "strains" (Brandel et al., 2003).

1.11 Cervid *PRNP* allele variants and susceptibility to CWD

The *PRNP* gene is highly conserved in the *cervidae* family; however, species-specific allele variants have been identified to code for PrP^C molecules with different amino acid sequences (Figure 1.3). In white-tailed deer populations affected by CWD, the most common *PRNP* allele (wt) codes for glutamine (Q), glycine (G), alanine (A) and glutamine (Q) at amino acid 95, 96, 116 and 226 respectively (wt-PrP^C = $Q^{95}G^{96}A^{116}Q^{226}$). Various *PRNP* alleles coding for amino acid substitutions have been identified; 95 (Q/H), 96 (G/S), 116 (A/G), 226 (Q/K), 226 (Q/R) and 230 (Q/L) (Johnson et al., 2003; 2006a; O'Rourke et al., 2004; Kelly et al., 2008; Wilson et al., 2009; Brandt et al., 2015).

PRNP allelic frequencies suggest deer expressing S96-PrP^C exhibit resistance to CWD compared to those expressing wt-PrP^C (Johnson et al., 2003; 2006a; O'Rourke et al., 2004; Kelly et al., 2008; Wilson et al., 2009; Keane et al., 2008; Brandt et al., 2015).

Experimental infection of white-tailed deer expressing Q95G96 (wt), Q95S96 (S96) or H95G96 (H95) PrP^C molecules confirmed the impact of *PRNP* polymorphisms on the progression of CWD (Johnson et al., 2011). Homozygous wt/wt deer were highly susceptible to CWD, succumbing to disease with an average survival of 23 months post-infection (mpi). Heterozygous deer S96/wt, H95/wt or H95/S96 had longer incubation periods suggesting partial resistance to disease (Johnson et al., 2011). Interestingly, the effect of the H95-PrP^C impacted disease progression the most; while heterozygous S96/wt deer had an average survival of 32 mpi, deer expressing H95-PrP^C survived for more than >50 mpi (Johnson et al., 2011).

Independent transmission studies supported the association of S96-PrP^C with resistance to CWD infection (Miller et al., 2012). Consistent with the results provided by Johnson and coworkers (2011), wt/wt deer succumbed faster to disease with an average survival of 22 mpi. The survival of S96/wt deer ranged between 26-39 mpi. Finally, two out of three S96/S96 deer died of unrelated illness after 31 mpi, and one animal was euthanized with CWD after more than 70 months post infection (Miller et al., 2012).

Transmission experiments in transgenic mice expressing different deer PrP^C molecules supported the association of S96-PrP^C with reduced susceptibility to CWD (Meade-White et al., 2007, Race et al., 2011). Transgenic mice expressing deer wt-PrP^C succumbed to prion disease after inoculation with CWD isolates from different cervid species. In contrast, mice expressing S96-PrP^C were resistant to CWD infection and showed no evidence of pathology after 600 days post infection (Meade-White et al., 2007, Race et al., 2011).

The most prevalent mule deer PrP^C amino acid sequence is identical to the wt-PrP^C from white-tailed deer; however, *PRNP* alleles exist that code for amino acid variation at position D20G (PrP^C signal peptide) and S225F (Jewell et al., 2005).

Surveillance in free-ranging populations revealed that wt/wt mule deer were more likely to be infected than those expressing F225-PrP^C (Jewell et al., 2005). Jewell and coworkers also described previously unpublished data of experimentally infected captive mule deer from two retrospective studies. In two groups of mule deer, wt/wt deer succumbed with CWD between 19 to 23 mpi while heterozygote animals developed CWD after 36 mpi suggesting that deer possessing F225-PrP^C have reduced susceptibility to CWD (Jewell et al., 2005). More recently, differences in clinical presentation and histopathological features were described in F225/F225 deer compared to wt/wt deer (Wolfe et al., 2014). Unlike wt/wt mule deer and despite developing signs of CWD, mule deer expressing F225-PrP^C were negative for PrP^{CWD} aggregates in lymph nodes and obex. Further, the obex region of F225/F225 deer presented spongiform change with no detectable PrP^{CWD} (Wolfe et al., 2014).

The amino acid sequence of PrP^C in rocky mountain elk differs from deer at amino acid 226, expressing glutamic acid (E) instead of glutamine (Q)(Cervenáková et al., 1997). Another source of primary structure variation includes *PRNP* alleles coding for methionine (M) or Leucine (L) at position 132 of PrP^C (O'Rourke et al., 1999). Elk expressing M132-PrP^C were over-represented in free ranging and captive CWD affected animals from various locations (O'Rourke et al., 1999). However, a more recent surveillance study in samples from Colorado indicated the three 132 genotypes M/M, M/L and L/L are equally susceptible to CWD infection (Perucchini et al., 2008).

Experimental CWD transmission in elk expressing the three 132-*PRNP* genotypes resulted in differential CWD progression (Hamir et al., 2006b). Elk homozygous for methionine were euthanized with clinical symptoms at 23 months post-infection, followed by leucine (L) heterozygotes at 40 mpi. Three out of four homozygous L132/L132 elks developed clinical disease at 59, 63 and 64 mpi; the fourth elk was healthy at the time of euthanasia (Hamir et al., 2006b; O'Rourke et al., 2007). In addition to the extended incubation times, L132/L132 elk presented a distinct protease resistant PrP^{CWD} pattern compared to the M132/M132 and M132/L132 elk (O'Rourke et al., 2007).

Transgenic mice expressing different M132-PrP^C molecules have differential susceptibility to CWD. After inoculation with elk and mule deer CWD isolates, only mice expressing the M132 PrP^C molecule succumbed to the disease with prion pathology (Green et al., 2008). Mice expressing L132 PrP^C appeared resistant at >600 days post infections and displayed no evidence of prion pathology.

In moose, two *PRNP* alleles have been reported to code for PrP^C molecules with different amino acid sequences at position 209(M/I) (Huson and Happ., 2006). CWD cases in moose are less abundant than in other cervids and only a few cases in free-ranging moose appear in the literature; one of these cases was homozygous for M209 (Baeten et al., 2007). The existing information is not enough to define the effect of PrP^C amino acid variation on the susceptibility of moose to CWD infection. During experimental CWD infection of M209 homozygous and I209 heterozygote moose, abnormal PrP aggregates occurred in brain and lymph tissues at 15.5 and 18.9 mpi respectively; however, it is not possible to draw conclusions about the effect of PrP^C

primary sequence as these animals died without CWD clinical symptoms (Kreeger et al., 2006).

The relative abundance of these two *PRNP* alleles and their effect on CWD susceptibility and

progression remains to be determined.

Rangifer tarandus sp is present in Northern Europe (European reindeer) and North America

(Barren ground caribou, woodland caribou, Porcupine caribou). Various PrP^C polymorphisms

have been identified in european reindeer; 129 (G/S), 138 (S/N), 169 (V/M), 176 (N/D), 225 (S/Y)

(Mitchell et al., 2012; Wik et al., 2012). In North America, *PRNP* alleles have been described to

change the primary structure of caribou PrP^C (Happ et al., 2007). In three caribou herds from

Alaska, five *PRNP* alleles code for different PrP^C amino acid polymorphisms (Happ et al., 2007).

Experimental CWD infection of reindeer of the various *PRNP* genotypes confirmed the

susceptibility of this cervid to CWD (Mitchell et al., 2012). This transmission experiment showed

that variation at position 138 (S/N) impact CWD susceptibility. Homozygous S138-*PRNP* animals

developed CWD after 18 and 20 mpi. Heterozygous N138-*PRNP* reindeer resisted CWD

infection, no accumulation of PrP^{CWD} was detected at >60mpi (Mitchell et al., 2012). The *PRNP*

allelic composition of other caribou subspecies from North America remains to be determined.

1.12 The species or transmission barrier

The interactions between the prion strain and the host PrP^C molecules modulate the ease of

transmission or the susceptibility to develop lethal disease. This interaction represents a primary

component of the transmission barrier (or species barrier during interspecies transmission) and is

of particular importance for studying the epizoology of prion diseases, including chronic wasting disease.

Extension of prion disease incubation periods can occur following transmission of a prion strain between two different host species or between individuals of the same species that express different PrP^C primary structures (e.g. different *PRNP* alleles coding for amino acid variations). Newly generated PrP^{Sc} and development of clinical signs is an indication that the prion agent is adapting to the new host. With one recent exception (Bian et al., 2017), the subsequent passages result in shorter and stable incubation periods if the transmission conditions remain constant (Bartz et al., 1994). Crossing the species or transmission barrier can lead to various outcomes that depend on the strain, the particular host and the infection status of the donor host (i.e. natural co-infection with more than one strain). These outcomes include mutation, reversible adaptation, selection or co-amplification of strains from a mixture (Bruce and Dickinson, 1987; Kimberlin et al., 1987; Kimberlin et al., 1989; Bruce, 1993; Bartz et al., 1994; Angers et al., 2010; Duque Vélasquez et al., 2015).

1.13 Biological stability of CWD strains

The 87A strain, derived by passage in mice of scrapie field isolates from different sheep breeds is one example of spontaneous mutation. The first passage of 87A sheep isolates in C57BL mice resulted in very long incubation periods that shortened and stabilized over subsequent passages. However, on certain occasions during these passages, a partial spontaneous shift would occur in a proportion of mice resulting in the accumulation of a new strain, 7D, with shorter incubation period and distinct neuropathology (Bruce and Dickinson, 1987).

The 22A strain, another scrapie strain isolated in mice, has been shown after numerous passages to adapt between host expressing different PrP^C molecules. However, when passaged into the mice used for isolation this strain regains its original properties (Kimberlin et al., 1989; Bruce, 1993). Some strains can follow this pattern when passaged between certain host species, but change into an irreversible state when moved to other host species. The strain 139A can be reversibly adapted between mice of different *Prnp* and between mice and rats, but when moved from any of these species into hamsters, it irreversibly changes into a unique strain (Kimberlin et al., 1987; Bruce, 1993). A similar behaviour characterized TME prions; reversible adaptation occurred between mink and ferrets (Bartz et al., 1994), however when TME was transmitted into hamsters, two strains (Hy and Dy) could be identified (Bessen et al., 1992a). Even after the TME agent was biologically cloned (by passages at low dose in mink), transmission into hamsters resulted in selection and adaptation of Hy and Dy (Bartz et al., 2000). Importantly, the emergence of new strains may involve changes in the host range. Mule deer CWD isolates have been shown incapable of transmitting to hamsters. However, when passaged through an intermediate species (i.e. ferret) the resulting strain can be passaged into hamsters (Bartz et al., 1998).

Interspecies transmission can also result in selection of different strains from a mixture. In instances when field isolates were transmitted, the donor host can be naturally co-infected with more than one strain resulting in hindered adaptation by the competition between these strains (Angers et al., 2010). Transmission of mule deer and elk CWD isolates into transgenic mice expressing deer PrP^C resulted in co-amplification of two CWD strains (CWD1 and CWD2) (Angers et al., 2009; 2010).

1.14 Chronic wasting disease strains

Numerous lines of evidence suggest diversity of CWD strains. As noted above, Angers et al., 2010 identified two strains, CWD1 and CWD2 in mule deer and elk. As outlined in the previous sections, particular mule deer CWD strains from Colorado are not able to produce prion disease in Syrian golden hamsters (Bartz et al., 1998). Further inoculation experiments achieved transmission of mule deer and elk CWD in hamsters after first passage, while CWD prions from white-tailed deer were not transmissible (Raymond et al., 2007). The transmission results in hamsters provide evidence of strain variation within mule deer and between mule deer, elk and white-tailed deer.

In the following chapters, I report on the biochemical and transmission properties of CWD strains (Wisc-1 and H95$^+$) derived from experimentally infected white-tailed deer. One of these strains (H95$^+$) represents a novel emergent CWD strain that differs in its transmission properties from Wisc-1 and CWD2. Unlike Wisc-1, H95$^+$ can infect transgenic mice expressing a common white-tailed deer resistance *PRNP* allele (Duque Vélasquez et al., 2015). Also and unlike CWD2, H95$^+$ produces disease in non-transgenic mice (i.e.C57BL/6) a species that has been shown to be resistant against previously tested CWD isolates (Raymond et al., 2007).

CHAPTER 2

CELLULAR PRION PROTEIN POLYMORPHISMS MODIFY THE SPECTRA OF CHRONIC WASTING DISEASE PRION CONFORMERS

Manuscript in preparation

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2.1 Abstract

Chronic wasting disease (CWD) in cervids involves the misfolding of cellular prion proteins (PrP^C) into infectious, strain-encoding protein structures (PrP^{CWD}) termed prions. Conversion and recruitment of host PrP^C into PrP^{CWD} aggregates sustains replication. Transmission of CWD within and between cervid species expressing PrP^C amino acid polymorphisms is thought to drive emergence and adaptation of strains with novel properties. We compared the biochemical and structural properties of deer CWD prions composed of different PrP primary structures. Cervid PrP^C polymorphisms introduced conformational modifications that affected the levels of PrP^{CWD} and the structural stability of PrP^{CWD} aggregates. Other properties such as the electrophoretic profile of PK-res PrP^{CWD} and a novel C-terminal PrP fragment were observed. Transmission of CWD prions in deer expressing PrP^C molecules with single amino acid differences generated PrP^{CWD} heterogeneity.

2.2 Introduction

Mammalian prions are unconventional infectious agents composed of misfolded, self-replicating conformations of the host encoded (i.e. locus *PRNP*) cellular prion proteins (PrP^C). The recruitment of PrP^C molecules into abnormal templating-PrP assemblies underlies prion propagation and brain accumulation of PrP aggregates during disease in various species (Prusiner, 1998; Gajdusek, Gibbs and Alpers, 1966; Vasquez-Fernandez et al., 2016). Unlike PrP^C, prion conformers and aggregates are insoluble in detergents, relatively resistant to proteases (i.e. proteinase K: PK) and protein denaturants (i.e GdnHCl) (Safar et al., 2015). Prions have been associated with epidemics of sheep "*Scrapie*", chronic wasting disease (CWD) in cervids, bovine spongiform encephalopathy (BSE) in cattle, and *Kuru*, variant and iatrogenic Creutzfeldt-Jakob

disease (vCJD and iCJD) in humans (Bruce, 2003; Wadsworth et al 2008; Angers et al., 2010). Different disease phenotypes within hosts expressing identical PrP^C molecules occur in concert with biologically distinct strains. Structural variants of disease-specific prion conformers (i.e. PrP^{CWD}, PrP^{Sc}, etc.) and the quaternary arrangements they form are thought to encode the epigenetic identity of each strain (Kasack et al., 1985; Bessen and Marsh, 1992b; Hill et al., 1997; Safar et al., 2015).

Natural transmission of prions occurs within and between species expressing different PrP^C primary structures. Following exposure to infectious material, the susceptibility of a given host to lethal disease is relative to the specific encountered strain and is associated with variable barriers to transmission (Bruce et al., 1994; Hill et al., 1997; Wadsworth et al., 2004). The route of exposure, the infectious dose and the structural compatibility between invading prions and the host PrP^C molecules contribute to the relative strength of these barriers, modulating incubation period, disease progression and lethality of the infection within the host lifespan (Prusiner et al., 1990; Bruce et al., 1991; Hill et al., 2000). Single PrP^C amino acid polymorphisms within hosts of the same species can differentially regulate susceptibility to conspecific or interspecific prion strains. Thus, while individuals of given *PRNP* genotypes succumb faster and more often, those expressing PrP^C allelic variants can show resistance and have extended incubation periods that may exceed their natural lifespan (Wadsworth et al., 2004; Johnson et al., 2011; Asante et al., 2015).

Propagation of strains across the transmission barrier of new host PrP^C primary structures can introduce reversible and permanent modifications in their biological properties (Kimberlin et al.,

1987; 1989; Bartz et al., 1998). The mechanism by which prions mutate and adapt to new hosts remains unresolved. Strains are thought to exist as quasispecies of prion conformers, which are maintained under selection on the basis of their compatibility with the host genetic and cellular background (i.e. *PRNP*). Under this model, mutant prion strains or substrains emerge by selection of conformational subspecies compatible with the new host PrP^C primary structures (Bruce et al., 1992; Bartz et al., 2000; Collinge, 2016). Emergent strains can have novel transmission properties that favor adaptation in other host species (Bessen and Marsh, 1992a; Bartz et al., 1998; Duque Velásquez et al., 2015). Defining the origin of emergent strain properties as induced or permitted by the host is limited when analyzing field isolates, as many hosts can be naturally co-infected with different strains (Bruce et al., 1992; Angers et al., 2010; Crowell et al., 2015).

The transmission cycle of CWD within cervid species is promoted by host-to-host interactions and environmental persistence of infectivity released in secretions, decomposing tissues and carcasses of diseased cervids (Miller and Williams, 2003; Saunders et al., 2012; Henderson et al., 2015). In populations where CWD is enzootic, transmission of prions between hosts expressing variable PrP^C primary structures (*PRNP* allelic variants) is thought to modulate the diversity of CWD strains (Angers et al., 2010; Angers et al., 2014; Duque Velasquez et al., 2015). PrP^C amino acid polymorphisms at residue 95 (Q/H) and 96 (G/S) regulate the susceptibility of white-tailed deer (*Odocoileus virginanus*) to CWD (Johnson et al., 2006a, 2011; Miller et al., 2012). Likewise, expression of these PrP^C variants in transgenic mice recapitulates the effects on susceptibility against various cervid prions (Meade-white et al., 2007, Race et al., 2011). The transmission barrier imposed by S96-PrP^C has been associated with disease driven changes in the S96-*PRNP* allelic frequency of chronically exposed cervid populations (Robinson et al., 2012a). It is unclear

how these polymorphisms interact with circulating CWD strains to modulate susceptibility to disease, structural variability and host selection of prion conformers.

Prion strains can be distinguished based on the properties of their specific abnormal PrP conformers and aggregates (Kasack et al., 1985; Bessen and Marsh 1992b; Capobianco et al 2007). Their size and mass, glycosylation status and stability in chemical denaturants (i.e. GdnHCl or SDS) vary between strains (Kasack 1985; Ayers et al., 2011; Safar et al., 2015).

To determine the effects of prion replication in hosts expressing different PrP^C primary structures, we examined the biochemical and structural properties of deer CWD prions composed of H95G96 (H95), Q95S96 (S96) or (wt) Q95G96-PrP molecules (Figure 2.1). Importantly, all these deer were infected with the same parental prion isolate (Wisc-1). Western-blot (WB), conformational dependent immunoassay (CDI), conformational stabiliy immunoassay and resistance to proteolysis were used to compare the protease sensitive (sen) and resistant (res) PrP^{CWD} fractions that compose these CWD prions.

2.3 Materials and methods

2.3.1 Brain homogenates

Four CWD agents consisting of 10% or 1% (wt/vol) brain homogenates (Bh) in phosphate-buffered saline were used for these studies (Johnson et al., 2011). CWD brain tissue was obtained from orally infected white-tailed deer expressing different PrPC molecules: homozygous Q95G96 (wt/wt), heterozygous Q95S96/wt (S96/wt), heterozygous H95G96/wt (H95/wt), and H95G96/Q95S96 (H95/S96) (Johnson et al., 2011). Frozen sagittal brain halves were

homogenized (blended) to 20% (wt/vol) in cold phosphate buffer (1.3M NaCl, 70mM Na$_2$HPO$_4$·2H$_2$O, 30mM NaH$_2$PO$_4$·2H$_2$O, pH 7.4), aliquoted, and stored at -80°C. Subsequently, aliquots were homogenized in a 50-ml syringe by passage through needles of different sizes (18 gauge to 21 gauge).

2.3.2 SDS-PAGE and western blot

Brain homogenate protein content was determined using a micro-bicinchoninic acid assay kit (Life Technologies). For the proteinase digestion reactions, 100 µg total protein (final volume 70 µl) was treated with 150 µg/ml of proteinase K (Life Technologies) for 45 min at 37°C. For removal of carbohydrates, approximately 50 µg of total protein were deglycosylated with 1500 Units of PNGase F (New England Biolabs) in a final volume of 70 µl of water. Enzymatic reactions were terminated by boiling the samples in 2.5X Laemmli buffer (150 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 25% glycerol, 5% [wt/vol] SDS, 12.5% ß-mercaptoethanol) at 95°C for 10 min. Samples (10 to 15 µg) were resolved on 12% NuPAGE bis-Tris gels (Life Technologies) and transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore). The membranes were blocked for 1 h at room temperature with 5% (wt/vol) non fat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST). Detection was performed using primary monoclonal antibody BAR224 (0.2 µg/ml diluted 1:10,000 in 5% [wt/vol] nonfat dry milk in TBST; Bertin Pharma), 8G8 (0.2 µg/ml diluted 1:2,000; Bertin Pharma), 12B2 (0.2ug/ml diluted 1:20.000; Wageningenur), 8H4 (0.2 µg/ml diluted 1:10.000; Abcam). Development was carried with secondary horseradish peroxidase-conjugated goat anti-

mouse IgG antibody, and chemiluminescence substrate (diluted 1:10,000; Life Technologies).

Images were acquired on X-ray film (Super Rx; Fujifilm).

2.3.3 Protease digestion time course

Brain homogenates (100 µg total protein) were incubated with 100 µg of proteinase K (or

thermolysin) (final concentration of sample protein to PK (or thermolysin) ratio, 1 to 1 mg/ml).

Samples were incubated at 37°C (70°C for thermolysin) and from each reaction aliquots were

removed every two hours for the first 12 hours. Additional samples were taken after 24 and 48

hours of incubation. Upon removal of the aliquots, the samples were boiled in 2.5X Laemmli

buffer (150 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 25% glycerol, 5% [wt/vol] SDS,

12.5% ß-mercaptoethanol) at 95°C for 10 min. The samples were resolved by SDS-PAGE and

western blot as previously mentioned. Quantification of PrP-res signals was performed using

ImageJ soft-ware (NIH). Three independent enzymatic digestions were performed. The signal

intensity from each replicate was averaged and plotted.

2.3.4 Conformation dependent immunoassay (CDI)

CDI analysis was performed, as described previously by the Safar lab (Safar et al., 1998;

Haldiman et al., 2013). Brain homogenate samples (5% w/v made in 1% v/v Sarkosyl in PBS, pH

7.4) were divided into two aliquots: one was left untreated (native) and the other was mixed with a

final concentration of 4M GdnHCl and heated for 5 minutes at 80°C (denatured). Both samples

were then diluted 20-fold in H2O containing protease inhibitors (PMSF, aprotinin and leupeptine

at 4µg/ml each) and 0.007%(v/v) of Patent blue V (Sigma). Aliquots of 20 µl were loaded on 96-

well Lumitrac high binding plates (E&K Scientific) prefilled with 200 µl of assay buffer (PerkinElmer Life Sciences). The plates were previously coated with monoclonal antibody 8H4 (epitope 175-185) in 200 mM NaH_2PO_4 containing 0.03% (w/v) NaN, pH 7.5. Following sample addition, the plates were incubated for 2 hours and then blocked with Tris buffer saline (TBS) pH 7.8 containing 0.5% BSA (w/v) and 6% sorbitol (w/v) for 1 hour at room temperature. The plates were then washed in TBS containing 0.05% (v/v) Tween-20. The captured PrP was detected with europium-conjugated 12B2 (amino acids 93-97 cervid numbering) or 8G8 (amino acids 100-105 cervid numbering). Plates were incubated with respective antibody for 2 hours washed with TBS-0.05% Tween-20 and developed with enhancement solution (Wallac inc). The time-resolved fluorescence (TRF) signal was measured with a multi-mode microplate reader (BMG Lab tech). The concentration of PrP was calculated from the CDI signal using a calibration curve prepared with either recombinant PrP (23-231) for samples containing full length PrP^{CWD} or with recombinant PrP (90-231) for samples containing truncated PK-res PrP^{CWD}. The TRF signal of the native sample corresponds to the 12B2 or 8G8 epitopes exposed in PrP^C but hidden in PrP^{CWD} and that are proportional to the concentration of PrP^C (Safar et al., 1998). The TRF signal of the denatured aliquot corresponds to the total PrP in the sample and the concentration of PrP^{CWD} was calculated according to the following: $[PrP^{CWD}] = [PrP\ denatured] - [PrP\ native]$. The concentration of PK-res PrP^{CWD} was calculated for samples treated with proteinase K followed by denaturation in GdnHCl. The concentration of PK-sen PrP^{CWD} was calculated accordingly: $[PK\text{-}sen\ PrP^{CWD}] = [PrP^{CWD}] - [PK\text{-}res\ PrP^{CWD}]$.

2.3.5 Structural stability assays

Assessment of structural stability was performed as follows (Safar et al., 1998; Haldiman et al., 2013). Frozen brain homogenate aliquots containing PrP^{CWD} were thawed and sonicated three times for 5 seconds at 60% amplitude with a sonicator 4000 (Qsonica) and the concentration was adjusted to approximately 50 ng/ml PrP^{CWD}. Aliquots of 15 µl contained in 15 microfuge tubes were treated with increasing concentrations of 8M GdnHCl containing 0.007% (v/v) Patent blue V (sigma) in 0.25M or 0.5M increments. Samples were incubated for 30 minutes at room temperature and rapidly diluted with assay buffer (Perkin Elmer) containing diminishing concentrations of 8M GdnHCl to a final concentration of 0.411M for all samples. Each aliquot was loaded into Lumitrac high binding plates coated with 8H4 antibody and developed according to the CDI method using antibodies 12B2 or 8G8 labeled with europium.

The TRF signal was converted into apparent fractional change of unfolding (F_{app}) according to the equation $F = (TRF_{OBS} - TRF_N) / (TRF_U - TRF_N)$, where TRF_{OBS} is the observed TRF value, and TRF_N and TRF_U are the values for native and unfolded forms at a given concentration of GdnHCl. To establish the concentration of GdnHCL where half of the PrP^{CWD} is denatured ($[GdnHCl]_{1/2}$), the data is fitted by a least square method with a sigmoidal transition model (Equation 1),

Equation 1: $$F_{app} = F_0 + \frac{(F_{max} - F_0)}{1 + e^{\{(C_{1/2} - C)/r\}}}$$

The apparent fractional change (F) in the TRF signal is the function of GdnHCl concentration (c); $c_{1/2}$ is the concentration of GdnHCl where 50% of PrP^{CWD} is dissociated/unfolded, and r is the slope constant.

The effect of protease treatment on the stability of PrP^{CWD} was established by subtraction of the fractional change values after PK treatment, from the F_{app} values obtained before PK treatment ($\Delta F_{app} = F^0 - F^{PK}$). This value was the fitted with a Gaussian model to estimate the proportion and average stability of PK-sen PrP^{CWD} conformers (Equation 2),

Equation 2: $$\Delta F_{app} = F_0 + A^{\{-(C-C0)2\}}$$

In this model, the PK-induced fractional change is ΔF; F_0 is the fractional change at 0 Molar GdnHCl, and c_0 is the GdnHCl concentration at the maximum height A of the peak.

2.4 Results

2.4.1 Analysis of PrP species in brain homogenates from CWD-infected white-tailed deer.

To facilitate the comparison of deer prions of different PrP^C primary structures, detection of PrP molecules was achieved with a panel of monoclonal antibodies (12B2, 8G8, BAR224 and 8H4). The specific PrP epitopes and their location in the structure of PrP are presented (Figure 2.1). To evaluate the effect of these polymorphisms in the PK-res PrP^{CWD}, brain homogenates were treated with PK and resolved by SDS-PAGE and western blot. Analysis revealed that H95/wt and S96/wt

brain homogenates contained lower levels of PK-res PrP^{CWD} compared to those from wt/wt deer. Unlike PK-res PrP^{CWD} from deer expressing wt-PrP only, expression of both polymorphisms H95 and S96, which regulate incubation period in deer, disrupted detection of PrP^{CWD} by monoclonal antibody 12B2 (N-terminal epitope 94-98) (Figure 2.2). Antibodies for alternative epitopes (Figure 2.1) revealed variation in the molecular mass and glycotype patterns of PK-res PrP^{CWD} molecules accumulated in the H95/S96 deer brain (Figure 2.2). The levels PK-res PrP^{CWD} in H95/S96 brain homogenates was lower than other samples and detection required higher (3X) brain protein μg equivalents compared to brains from other deer.

Figure 2.1. Graphic representation of deer PrP^C and of experimental white-tailed deer CWD isolates. A) Polymorphisms at amino acid 95 and 96 appear in red letters. Brackets denote the linear epitopes for monoclonal antibodies. B) Origin of the experimental CWD isolates used for these studies (Johnson et al., 2011).
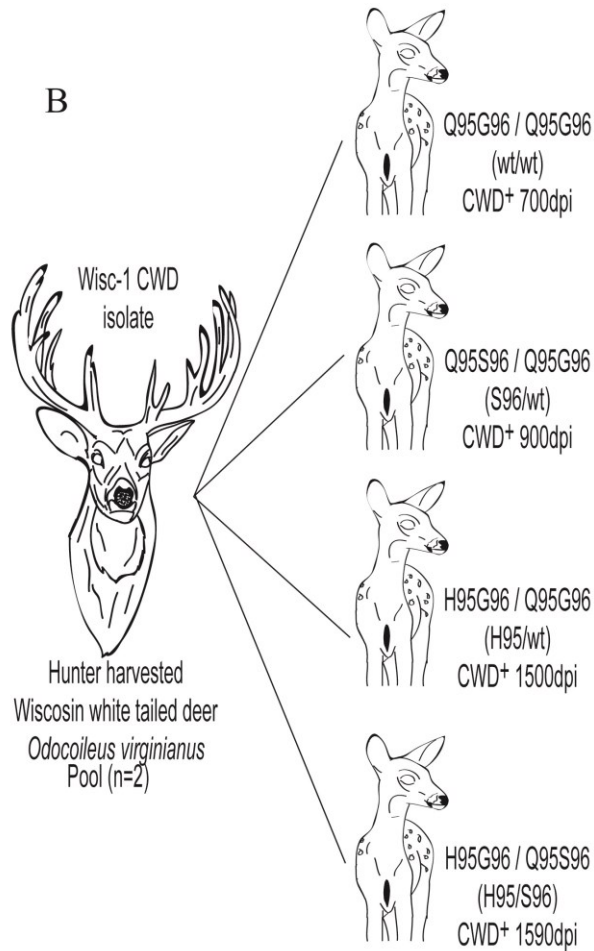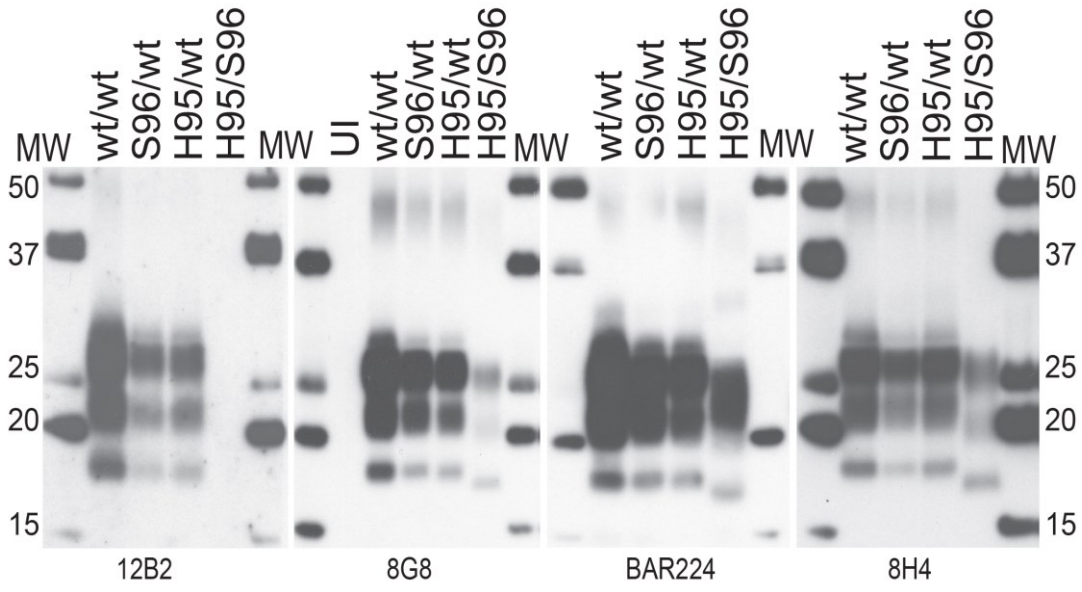
A
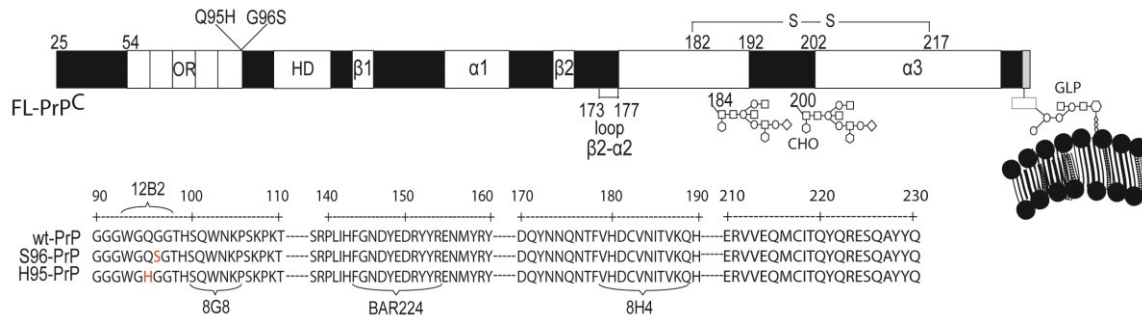
B

Figure 2.2. PK-res PrP^{CWD} in the brain of white-tailed deer expressing different PrP^C primary structures. Brain homogenates were treated with proteinase K (150 µg/ml) and resolved by SDS-PAGE and western blot. Detection was achieved with monoclonal antibodies (12B2, 8G8, BAR224 and 8H4). UI: uninfected.

To evaluate if the detection effects of the H95 and S96 polymorphisms were related to loss of the 12B2 epitope by the action of PK, brain homogenates were compared in the absence of protease treatment. Analysis of deer H95/S96 prions with antibody 12B2 resulted in negative detection of PrP^C and total PrP^{CWD} species (Figure 2.3). When compared with other antibodies, the total PrP (i.e. absent exogenous proteinase) species in the brain of deer H95/S96 included additional electrophoretic signals (Figure 2.3), indicating differences in glycosylation and/or N-terminal physiological cleavage of PrP^{CWD}.

Deglycosylation of the brain homogenates with PNGase F facilitated the identification of alternate PrP cleavage products present in H95/S96 brain homogenate (Figure 2.4). This C-terminal PrP fragment (C3-PrP) was approximately 20KD and present only in H95/S96 deer. The C2-PrP fragment in H95/S96 was also more abundant than in other deer (Figure 2.4).

Figure 2.3. Total PrP in the brain of white-tailed deer expressing different PrP^C primary structures. Brain homogenates (in the absence of exogenous proteases) were resolved by SDS-PAGE and western blot and detected with monoclonal antibodies (12B2, 8G8, BAR224 and 8H4).
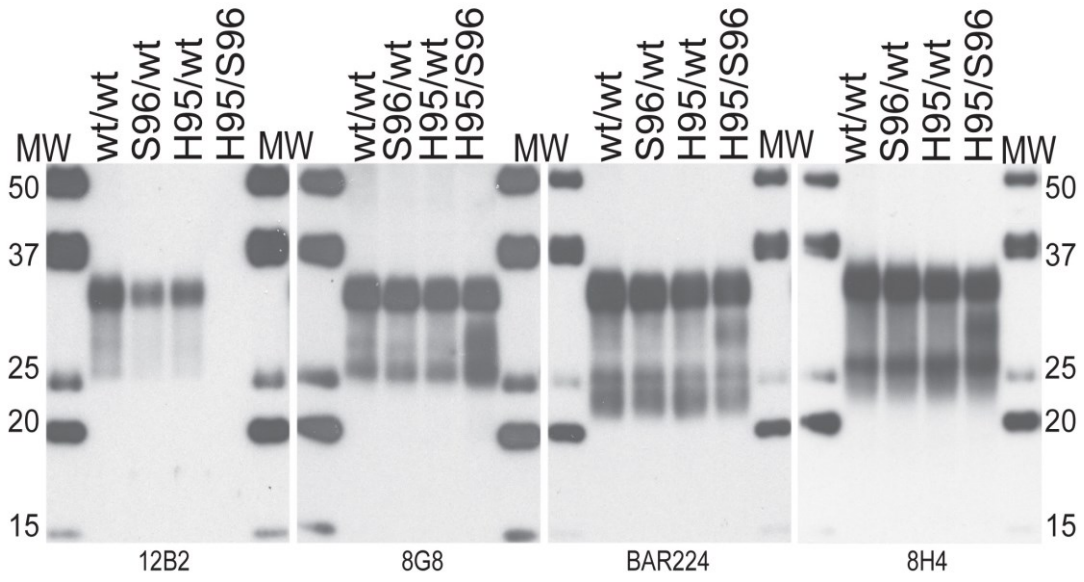
Figure 2.4. Deglycosylated total PrP in the brain of white-tailed deer expressing different PrP^C primary structures. Brain homogenates (in the absence of exogenous proteases) were treated with PNGase F (1500U) and evaluated by SDS-PAGE and western blot. Detection was performed with monoclonal antibodies (12B2, 8G8, BAR224 and 8H4).

MW | wt/wt | S96/wt | H95/wt | H95/S96 | MW | wt/wt | S96/wt | H95/wt | H95/S96 | MW | wt/wt | S96/wt | H95/wt | H95/S96 | MW

50
37
25 ← Full lenght
20 ← C3
← C2
15 ← C1

12B2   8G8   BAR224
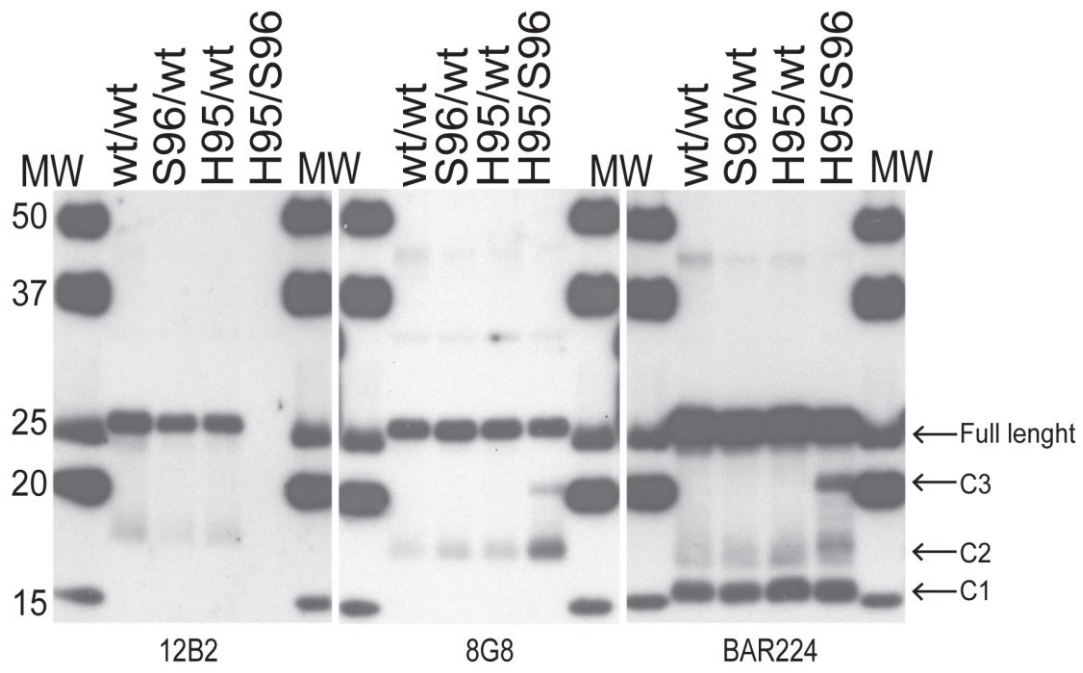
2.4.2 Structural resistance of deer PrP^{CWD} aggregates against proteolysis

To compare the relative resistance to proteolysis of these PrP^{CWD} aggregates, brain homogenates from these deer were treated with two proteases (Proteinase K and thermolysin) for different times. The resistance to proteolysis differed depending on the primary structure. While PrP^{CWD} prions in the brain of H95/S96 deer were most susceptible to digestion with proteinase K (PK) and thermolysin, prions derived from deer expressing wt-PrP^{C} showed similar responses to PK digestion and thermolysin (Figure 2.5).

2.4.3 Relative levels of PrP species in deer brain homogenates

Considering the structural and biochemical heterogeneity associated with H95 and S96-PrP^{CWD} variants, CDI was used to measure the relative abundance of PrP^{C}, PK-sen PrP^{CWD} and PK-res PrP^{CWD} molecules in brains of deer expressing different primary structures. Expression of S96-PrP^{C} or H95-PrP^{C} affected the accumulation of PrP^{CWD} species. When measured with 12B2-CDI, brain homogenates from H95/wt and S96/wt deer contained 56.8% and 68.6%, respectively, lower levels of total PrP^{CWD} (65 and 67% less PK-res PrP^{CWD}) than wt/wt deer brain homogenates (474 ng/ml of total PrP^{CWD} and 226 ng/ml of PK-res PrP^{CWD}) (Figure 2.6). The H95/wt and S96/wt CWD accumulated similar (78 and 74 ng/ml) amounts of PK-res PrP^{CWD} but contrasted in their content of protease sensitive PrP^{CWD} molecules (127 and 75 ng/ml respectively). CDI calibration with antibody 8G8 (N-terminal epitope 100-105) was required for measuring the content of total PrP^{CWD} species in H95/S96 deer brain homogenate. This deer accumulated lower levels of PrP^{CWD} than other deer (Figure 2.6). The content of PrP^{CWD} species (PK-sen and PK-res) in deer

expressing H95 and/or S96-PrP^C was lower compared to wt/wt deer, suggesting these PrP^C molecules are associated with an inhibitory effect upon infection with CWD.

Figure 2.5. PrP^{CWD} resistance to proteolysis. A) Brain homogenates were treated with proteinase K (PK 100 µg/ml) and sampled a different times. B) Time course of treatment with thermolysin (100 µg/ml). Following protease addition, aliquots were taken at 2, 4, 6, 8, 10, 12, 24 and 48 hours. Boiling in sample buffer stopped the protease digestion. Samples were resolved by SDS-PAGE and western blot. The signal intensity was quantified with image J. The average intensities (±standard deviation) of three experimental replicas for each CWD sample were plotted.

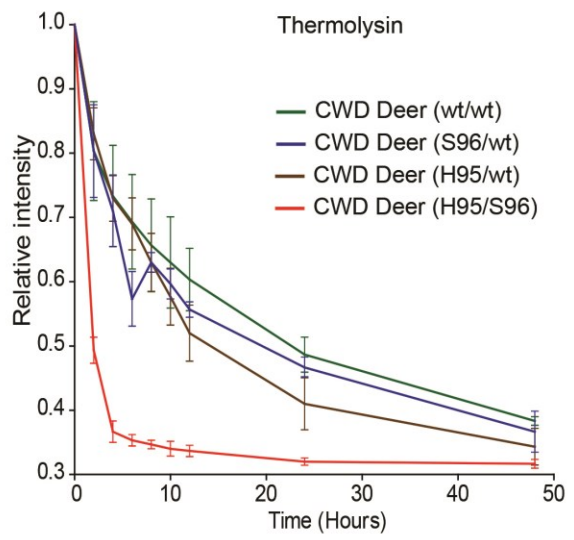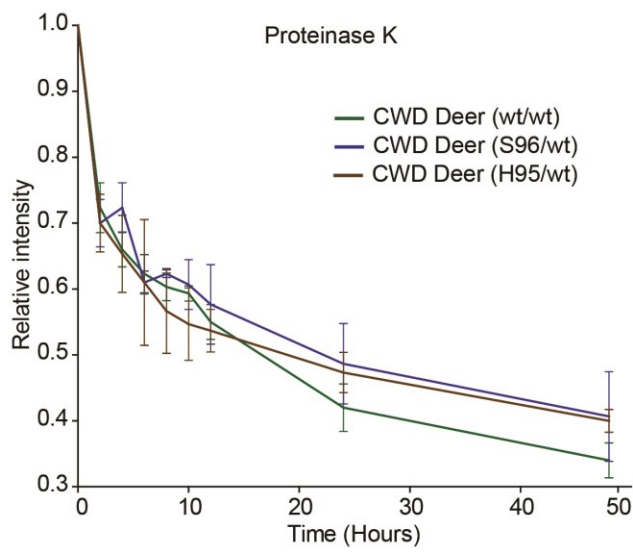**Figure 2.6.** Abundance of PrP species in brain homogenates from CWD-infected white-tailed deer. Brain homogenates were evaluated by conformation dependent immunoassay (CDI) before (-) and after (+) treatment with proteinase K. 8H4 was used as capture antibody and Europium-labeled 12B2 or 8G8 were used as detection antibodies. Data obtained in collaboration with Dr. Jiri Safar, Chae Kim and Tracy Haldiman.
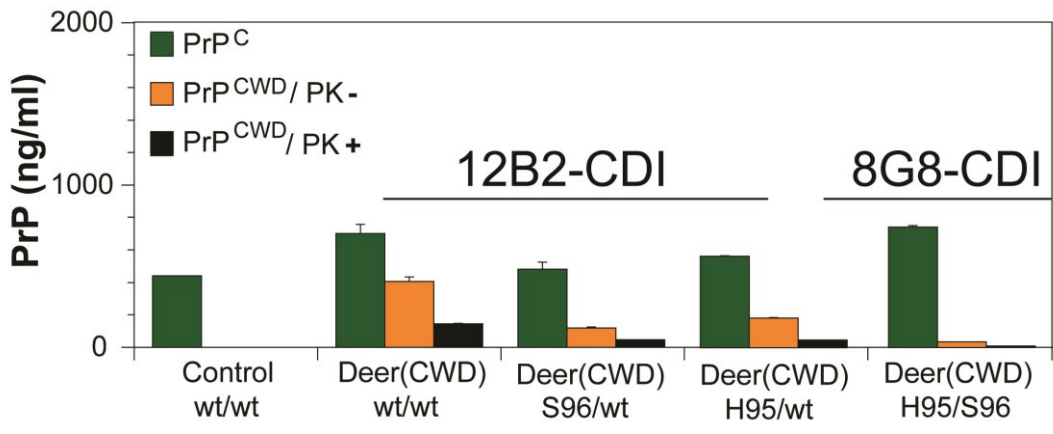
2.4.4 Structural stability of deer PrP^{CWD} in GdnHCl

Given the effects of deer PrP^{C} amino acid polymorphisms on structural variability, abundance and the properties of PrP^{CWD} molecules, the conformational stability of these CWD prion aggregates in increasing concentrations (0-4.5M) of GdnHCl was compared (Figure 2.7). Either 8G8 or 12B2-CDI was used to monitor the denaturation of allele-specific aggregates. Using 8G8-CDI in the absence of PK treatment, dissociation and unfolding of H95/S96 PrP^{CWD} structures occurred in two phases corresponding to low and high stability aggregates. S96/wt samples contained PrP^{CWD} species that resembled the denaturation pattern observed for low stability H95/S96 aggregates (Figure 2.7A). The average stability of H95/S96 and S96/wt PrP^{CWD} against GdnHCl was lower than wt/wt and H95/wt PrP^{CWD}, with denaturation midpoints at 2.0, 2.3, 2.6 and 2.7M respectively (Figure 2.7A). Following protease treatment, the wt/wt PK-res PrP^{CWD} aggregates were more susceptible to unfolding than those of S96/wt, H95/wt and H95/S96 deer (Figure 2.7B).

The differential reactivity of 12B2, which does not recognize the H95 and 96 prion proteins, allowed the examination of the PrP^{CWD} aggregates composed of wt-PrP. The profiles of wt/wt and S96/wt PrP^{CWD} assemblies and their GdnHCl denaturation midpoints shifted when dissociation and unfolding were monitored with 12B2-CDI (Figure 2.8A). However, following PK treatment the denaturation profiles overlapped (Figure 2.8B).

Figure 2.7. Dissociation and unfolding of PrP^{CWD} aggregates from deer expressing different PrP^C molecules monitored with monoclonal antibody 8G8. Brain homogenates were treated with increasing concentrations (M) of GndHCl and evaluated by CDI with antibody 8G8. A) In the absence of proteinase K (PK-). B) After treatment with PK (+).

A
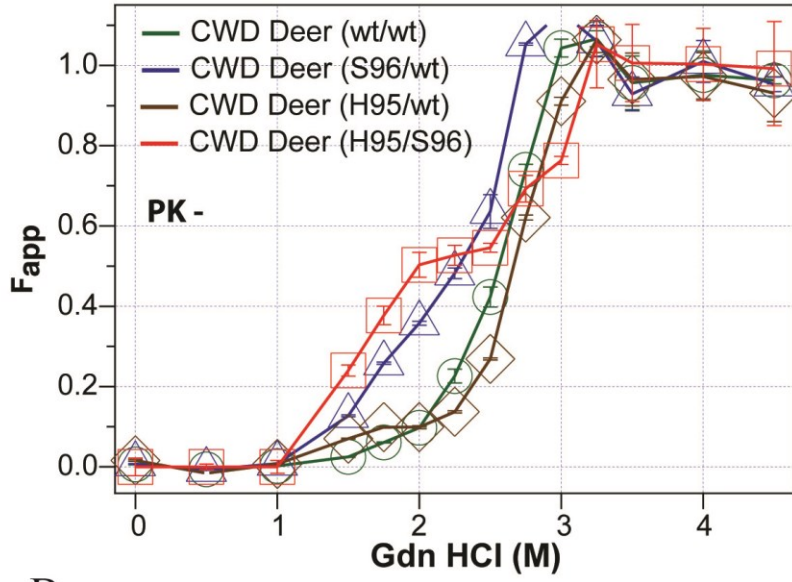
B

Figure 2.8. Dissociation and unfolding of PrP^{CWD} aggregates from deer expressing different PrP^C molecules monitored with monoclonal antibody 12B2. Brain homogenates were treated with increasing concentrations (M) of GndHCl and evaluated by CDI with antibody 12B2. A) In the absence of proteinase K (PK-). B) After treatment with PK (+).
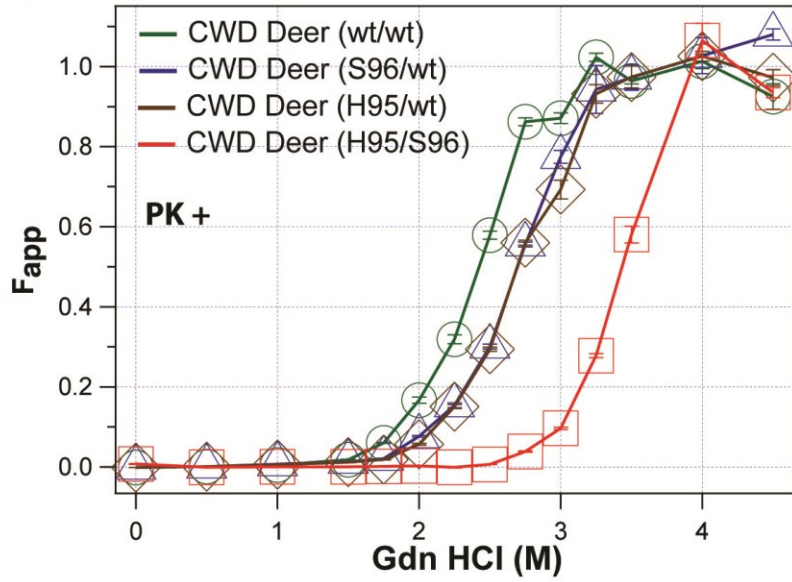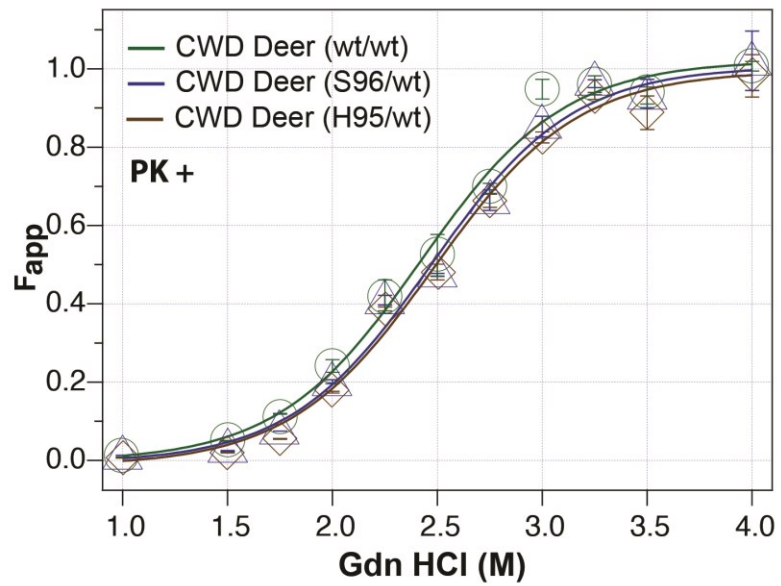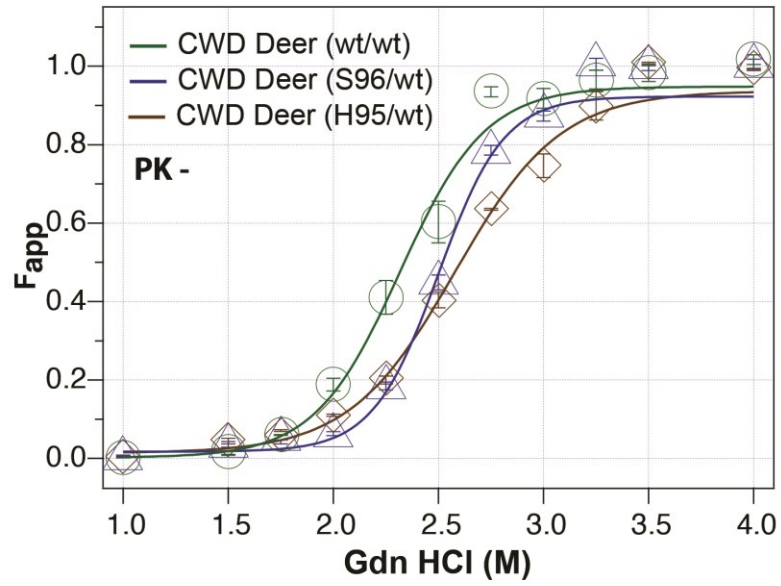
2.5 Discussion

Transmission of the Wisc-1 CWD isolate into deer expressing distinct PrP^C molecules resulted in variable PrP^{CWD} signatures. Besides the different disease progression (Johnson et al., 2011), the presence of H95-PrP^C or S96-PrP^C impacted various properties of the PrP^{CWD} molecules present in the brain of these deer. The deer expressing both polymorphisms accumulated PrP^{CWD} molecules with a distinct electrophoretic pattern.

Several examples exist in which different prion strains associated with dissimilar clinical phenotypes can produce PK-res PrP molecules with discernible migration patterns by SDS-PAGE and western blot. Examples include hamster-adapted TME prions (PrP^{Hy} and PrP^{Dy})(Bessen and Marsh, 1992b) and sporadic Creutzfeld-Jakob disease patients (PrP^{CJD1} and PrP^{CJD2}) (Safar et al., 2015). When compared to the wt/wt PK-res PrP^{CWD}, deer H95/S96 produced PK-res PrP^{CWD} with lower molecular weight and a different glycotype pattern suggesting the prion conformers present in this animal have a different potential to convert PrP^C molecules.

Although the H95/wt and S96/wt deer accumulated PK-res PrP^{CWD} molecules that resembled those of the wt/wt deer, examples exist of prions strains that are not readily differentiated by the gel migration of their PK resistant PrP products (Angers et al., 2010). Given the effects observed in the H95/S96 deer, it is possible that H95/wt and S96/wt deer also contain PK sensitive PrP^{CWD} molecules not accounted in the proteolysis profiles. Certain prion strains are more prone to degradation by proteases than others; this is the case for some PK-sensitive synthetic prions strains made from recombinant PrP expressed in bacteria (Colby et al., 2010). Also, Nor98, a strain from sheep, is characterized by high susceptibility to degradation by proteinase K compare to other scrapie strains (Pirisinu et al., 2013).

Expression of the H95 or S96 amino acids also affected detection of PrP^{CWD} with the 12B2 antibody. This difference is not related to alternative PK cleavages between the PrP^{CWD} molecules given that 12B2 does not recognize full-length H95-PrP^{CWD} or S95-PrP^{CWD}. In addition, 12B2 detection of H95-PrP^C or S96-PrP^C was impaired. These cervid polymorphisms are located in the center of the 12B2 epitope, at the edge of the octarepeats (OR) in the N-terminus of PrP^C. This epitope alteration suggests the steric effects of the histidine or serine side chains may introduce structural constraints that affect conversion of PrP^C into PrP^{CWD}, impacting deer susceptibility to CWD. In agreement with this proposition, the PrP amino acid polymorphisms affected the propagation of Wisc1 CWD in heterozygous deer, limiting the total production and accumulation of PK-sen and PK-res PrP^{CWD} molecules. The quantity of PrP^{CWD} in heterozygous deer was lower than that observed for deer expressing only (Q95G96) wt-PrP^C. The importance of the octarepeat domain has been evaluated previously. Transgenic mice expressing bovine PrP^C with a shorter OR are less susceptible to infection with BSE prions (Brun et al., 2007). Also, in humans, insertions or duplication of OR segments result in genetic forms of prion disease, but deletions of this regions have not been associated with increased susceptibility (Palmer et al., 1993). *In vitro* fibrilization studies with recombinant PrP molecules differing at a single amino acid have reported a dominant negative inhibition effect that results in lower yields of aggregation when both proteins are mixed (Lee et al., 2007).

A novel N-terminally truncated form of PrP (C3-PrP) was detected in the H95/S96 deer. In addition this animal presented more C2-PrP compared to other animals while the level of C1-PrP

was equivalent to other deer. N-terminal endoproteolysis of PrP^{Sc} and cell-specific accumulation of C-terminal PrP^{Sc} fragments differs between prion strains (Jeffrey et al., 2006a; Jeffrey et al., 2006b; Owen et al., 2007). A difference in N-terminal processing is suggested to represent prion strain specific susceptibility to clearance in neurons, which has been associated with variation of incubation periods (Ayers et al., 2011). Also, BSE infection in sheep is characterized by cell-specific N-terminally truncated PrP aggregates that differ from scrapie-infected sheep (Gonzalez et al., 2003). The alternative N-terminal fragmentation of PrP^{CWD} and the overproduction of C2-PrP may indicate the generation of heterogeneous PrP^{CWD} species following the passage of Wisc-1 in H95/S96 deer (Johnson et al., 2011; Duque Vélasquez et al., 2015). The similar molecular weight of C2 and C3-PrP with unglycosylated and monoglycosylated PK-res PrP^{CWD} indicates these fragments are PK resistant. C2-PrP is considered to be a naturally occurring version of unglycosylated PK-res PrP^{Sc} produced by the action of intracellular proteases (Chen et al., 1996).

Unfolding and dissociation profiles of heterozygous deer PrP^{CWD} assemblies in the presence of Guanidine hydrochloride (GndHCl) differed from wt/wt PrP^{CWD} aggregates. Variations in conformation stability have been shown between prion strains (Ayers et al., 2011; Safar et al., 2015). Compared to wt/wt PrP^{CWD} aggregates, the unfolding and dissociation spectra suggests that PrP^{CWD} aggregates in heterozygous deer are composed of different homotypic assemblies of both primary structures. Mixtures of independent PrP^{CJD} conformers have been previously shown to co-exist in the same patient (Haldiman et al., 2013). The possibility of these aggregates being composed of both proteins would require further characterization. However, it has been shown that GdnHCl unfolding of heterologous aggregates of different recombinant PrP do not differ from those composed by a single kind of PrP primary structure (Lee et al., 2007). Finally, changes in

the conformational stability of PrP^{Sc} have been associated with emergence of novel strain properties (Peretz et al., 2002).

Cervid PrP^{C} polymorphisms might affect the conformational evolution of CWD prion agents by 1) introducing structural modifications on the spectra of PrP^{CWD} conformers of invading prion strains, 2) restricting or narrowing the PrP^{CWD} conformational landscape (host selection) invading prions can adopt, resulting in selective propagation barriers that regulate emergence of CWD strains. The immediate consequences of PrP^{CWD} conformational heterogeneity are expansion of the CWD host range, resulting in CWD transmission into species considered resistant to infection (See chapters 3 and 4). Changes in host range can, in turn, expand the circulating strain pool affecting eradication programs, altering transmission dynamics in CWD enzootic populations and modifying host-pathogen co-evolution patterns (Robinson et al., 2012a). Finally, novel PrP^{CWD} conformers may escape detection methods, reducing the accuracy of disease surveillance.

CHAPTER 3.

DEER PRION PROTEINS MODULATE THE EMERGENCE AND ADAPTATION OF CHRONIC WASTING DISEASE PRION STRAINS.

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3.1 Abstract

Transmission of chronic wasting disease (CWD) between cervids is influenced by the primary structure of the host cellular prion protein (PrP^C). In white-tailed deer, *PRNP* alleles encode polymorphisms Q95G96 (*wt*), Q95S96 (*S96*) and H95G96 (*H95*) that differentially impact CWD progression. We hypothesize that transmission of CWD prions between deer expressing different allotypes of PrP^C modifies the contagious agent affecting disease spread. To evaluate the transmission properties of CWD prions derived experimentally from deer of four *PRNP* genotypes (*wt/wt, S96/wt, H95/wt* or *H95/S96*), transgenic (tg) mice expressing *wt* (tg33) or *S96* (tg60) alleles were challenged with these prion agents. Passage of deer CWD into tg33 mice resulted in 100% attack rates, with H95/S96 CWD having significantly longer incubation periods. Disease signs, neuropathological and PrP-res profiles in infected tg33 mice were similar between groups, indicating a prion strain (Wisc-1) common to all CWD inocula was amplified. In contrast, tg60 mice developed prion disease only when inoculated with the H95/wt and H95/S96 CWD allotypes. Serial passage in tg60 mice resulted in adaptation of a novel CWD strain (H95$^+$) with distinct biological properties. Transmission of first-passage tg60CWD-H95$^+$ isolates into tg33 mice, however, elicited two prion disease presentations consistent with a mixture of strains associated with different PrP-res glycotypes. Our data indicate that *H95-PRNP* heterozygous deer accumulated two CWD strains, whose emergence was dictated by PrP^C primary structure of the recipient host. These findings suggest CWD transmission between cervids expressing distinct PrP^C molecules results in generation of novel CWD strains.

3.2 Introduction

Chronic wasting disease (CWD) is an emerging prion disease or transmissible spongiform encephalopathy (TSE) of cervids, affecting free-ranging white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), elk (*Cervus canadensis*), and moose (*Alces americanus*) (Williams and Young, 1980; Baeten et al., 2007). CWD occurs in captive herds of these species in North America and in red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) in South Korea (Williams and Young, 1980; Thomsen et al., 2007; Lee et al 2013b). Reindeer (*Rangifer tarandus tarandus*), also known as caribou, are susceptible to experimental infection (Mitchell et al., 2012).

TSEs are slowly progressive, fatal neurodegenerative disorders for which no effective treatment or vaccine is available. Neuropathological changes include prion protein deposits, spongiform degeneration, neuronal loss, and astrogliosis. These hallmarks are diagnostic for CWD in cervids, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), as well as kuru, iatrogenic Creutzfeldt-Jakob disease (iCJD), and variant Creutzfeldt-Jakob disease (vCJD) in humans (Hadlow et al., 1980a; Gajdusek and Zigas et al., 1959; Wells et al., 1987; Duffy et al., 1974; Will et al., 1996).

The pathogenesis of TSEs is associated with misfolded prion protein (PrP^{Sc}; or PrP^{CWD} for cervid infections), whose ability to propagate, persist, and trigger neuropathology requires the expression of host *PRNP*-encoded cellular prion protein (PrP^{C}). Prion propagation involves the posttranslational misfolding of normal cellular PrP molecules into pathognomonic, transmissible,

generally proteinase K-resistant prion protein (PK-res PrP^{CWD}) conformers that progressively accumulate in brain and other tissues (Prusiner, 1982a, 1982b; Bolton, McKinley and Prusiner, 1982; Basler et al., 1986; Angers et al., 2006). The primary structure of PrP^C influences host susceptibility to infection, its disease progression, and its neuropathological and biochemical profiles (Dickinson et al., 1968a; Carlson et al., 1989; Bruce et al., 1991; Carlson et al., 1994; Bruce 2003; Meade et al., 2008; Brown et al 2012; Gonzalez et al., 2014; Wolfe et al., 2014). Knockout (*Prn-p*) mice are refractory to experimental infection with mouse-adapted scrapie (Bueler et al., 1993).

The difficulty of prion transmission from one species to another is defined as the species barrier, and that between individuals of the same species with different *PRNP* genotypes is defined as the transmission barrier and is influenced by the primary structure of the recipient's PrP^C (Dickinson et al., 1968a; Bruce et al., 1991; Bruce 2003; Meade et al., 2008, Prusiner et al., 1990; Lee et al., 2001). This barrier does not necessarily render the host refractory to infection and is impacted by the invading prion strain (Meade et al., 2008; Brown et al 2012; Lee et al., 2001; Wadsworth et al., 2004). Prions can exhibit strain diversity. Strains are distinguished on the basis of their host range, clinical presentation, disease progression, neuropathological and PrP^{Sc} biochemical profiles (Bruce and Dickinson, 1987; Bessen and Marsh, 1992; Capobianco et al., 2007; Griffiths et al., 2010). The propagation of prion strains is dependent on both the *PRNP* genotype of the recipient and the properties of the invading agent (Wadsworth et al., 2004; Dickinson and Fraser, 1979). For example, sheep expressing the V136-R154-Q171 PrP^C (GenBank accession number AJ567988) are most susceptible to classical scrapie, while sheep with distinct *PRNP* genotypes have reduced susceptibility (Le Dur et al., 2005; Moum et al., 2005; Saunders et al., 2006). The

strain of the agent also plays a role, as sheep expressing A136-R154-R171 PrP^C (GenBank accession number AJ567985) or A136-H154-Q171 PrP^C (GenBank accession number AJ567983) are susceptible to atypical scrapie, although they are relatively resistant to classical scrapie (Griffiths et al., 2010; Gotte et al., 2011). Similarly, the PrP^C primary structure and the invading agent modulate human susceptibility to prion infection; polymorphisms at amino acid 129 affect susceptibility to vCJD, kuru, and iCJD (Meade et al., 2008; Brown et al 2012; Lee et al., 2001; Wadsworth et al., 2004; Bruce et al., 1997; Ironside et al., 2006; Meade et al., 2007; Kaski et al., 2009), while the G127V mutation renders carriers resistant against kuru (Asante et al., 2015).

In regions of North America where CWD is enzootic, transmission occurs between cervids expressing heterologous PrP^C molecules (PrP^C allotypes [Carlson et al., 1994]). Analysis of *PRNP* allelic frequencies in wild and captive white-tailed deer identified two PrPC polymorphisms, Q95H and G96S, that impact susceptibility to CWD (Johnson et al., 2003; O'Rourke et al., 2004; Johnson et al., 2006a) (GenBank accession numbers AF156185, AF156184, and AY275711). Homozygous wild-type (wt; Q95 G96) deer are most susceptible to CWD and have relatively short incubation periods. In contrast, deer heterozygous for the S96/wt, H95/wt, and H95/S96 alleles had extended incubation periods, suggesting that S96-PrP^C and H95-PrP^C impact CWD prion propagation (Johnson et al., 2011). Miller et al. (2012) reported similar observations in experimentally challenged S96/wt and S96/S96 deer when the incubation periods for those deer were compared to those for wt/wt white-tailed deer and mule deer. To further explore the diversity of CWD strains and the consequences of propagation in deer expressing different PrP^C primary structures, brain homogenates from CWD-infected white-tailed deer of different *PRNP* genotypes (wt/wt, S96/wt, H95/wt, or H95/S96 [Johnson et al., 2011]) were inoculated into transgenic (tg)

mice expressing the deer wt or S96-PrP^C (Meade-White et al., 2007; Race et al., 2011). Our data show that CWD prions passaged in deer expressing H95-PrP^C have altered transmission properties. The H95/wt and H95/S96 CWD allotypes efficiently triggered prion disease in tg mice with *S96-PRNP* genotypes, leading to the identification and adaptation of a novel CWD strain. Transmission of first-passage tg60CWD-H95$^+$ prions into tg33 mice resulted in two distinct prion disease phenotypes, which resembled those observed after primary passage of H95-PrP heterozygous deer CWD in both tg lines.

3.3 Materials and methods

3.3.1. Deer CWD inocula.

Four CWD agents consisting of 10% or 1% (wt/vol) brain homogenates (Bh) in phosphate-buffered saline were used for transmission studies (Johnson et al., 2011). The inocula were designated on the basis of their specific *PRNP* genotypes. CWD brain homogenates were obtained from orally infected white-tailed deer expressing different PrP^C molecules: homozygous Q95 G96 (wt/wt), heterozygous Q95 S96/wt (S96/wt), heterozygous H95 G96/wt (H95/wt), and H95 G96/Q95 S96 (H95/S96) (Johnson et al., 2011). Brain homogenate from an uninfected white-tailed deer was used as a negative control. Frozen sagittal brain halves were homogenized (blended) to 20% (wt/vol) in cold phosphate buffer (1.3M NaCl, 70mM Na$_2$HPO$_4$·2H$_2$O, 30mM NaH$_2$PO$_4$·2H$_2$O, pH 7.4), aliquoted, and stored at -80°C. Subsequently, aliquots were homogenized in a 50-ml syringe by passage through needles of different sizes (18 gauge to 21 gauge).

3.3.2. Transmission studies in transgenic mice.

Animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Health Sciences Animal Care and Use Committee of the University of Alberta Animal Care and Use Committee. Bioassays were performed with transgenic mouse lines expressing the deer *wt-PRNP* allele (tg33$^{+/+}$ and tg33$^{-/+}$ mice) or the *S96-PRNP* allele (tg60 mice, which express 30% less PrPC than tg33$^{+/+}$ mice) (Meade-White et al., 2007; Race et al., 2011). Weanling pups were inoculated intracerebrally with 30 µl of deer CWD brain homogenates. Animals were monitored for the appearance of clinical signs and disease progression. Individual incubation periods are expressed as the number of days postinoculation (dpi) and were calculated from the time the mice were inoculated until the time that clinical disease was established. The distribution of incubation periods between groups of tg33 mice was compared using the Kruskal-Wallis test with Dunn's multiple-comparison posttest (P < 0.05). Survival times post-inoculation between tg60 mice inoculated with the H95/wt deer CWD allotype and the H95/S96 deer CWD allotype were compared using the Mann-Whitney test (P < 0.05). The statistical analysis of transmission experiments was performed with GraphPad Prism (version 5.04) software.

Isolates derived from tg60 mice infected with the H95/wt or H95/S96 deer CWD allotypes (tg60CWD-H95/wt and tg60CWD-H95/S96 isolates, respectively) were used for syngeneic and allogeneic passages. One tg60CWD-H95/wt isolate (10%, wt/vol) was transmitted in tg33 and tg60 mice. A tg60CWD-H95/S96 isolate (10%, 1%, 0.0001%, wt/vol) was also passaged into both tg lines.

3.3.3. Histopathological analysis.

Brain tissues from at least 5 (range, 5 to 11) tg mice per inoculum group were formalin fixed and paraffin embedded for histopathological analysis. Sagittal brain sections were obtained from 2 to 4 mice in each group of animals receiving each inoculum, and coronal brain sections were obtained from 3 to 7 mice in each group. Six consecutive slides of both sagittal and coronal brain sections (4 coronal sections from each brain) were examined as follows: 2 slides (4 to 6 μm thick) were stained with hematoxylin and eosin (H&E) to evaluate the sections for spongiform degeneration, and the other 4 slides were immunostained for PrP^{CWD} deposition and glial fibrillary acidic protein (GFAP)-positive astroglia. The sagittal paramedian brain sections were 0.36 to 0.60 mm lateral from the brain midline. All immunostaining experiments included CWD-positive tissue and negative mock-infected control sections. Differences in PrP^{CWD} deposition patterns could exist in areas of the central nervous system that were not examined. For the purpose of comparison, identification of the structures in H&E-stained slides was performed according to the mouse brain atlas (Franklin and Paxinos, 2007).

Lesion profile analysis was performed using coronal brain sections as described previously (Fraser and Dickinson, 1968). The lesion profile scores for the first passage in tg33 mice were obtained from 3 to 7 mice per inoculum group. For the CWD-affected tg60 mice, lesion profiles were obtained by scoring 4 mice per inoculum group. The density of spongiform lesions in nine gray matter areas from the brains of prion disease-affected mice were scored by three independent observers in a blind manner. The scores are reported as the mean ± standard deviation.

PrP^{CWD} deposits were visualized by immunostaining using anti-PrP monoclonal antibody

BAR224 or 8G8 (0.2 µg/ml diluted 1:2,000 or 1:100, respectively; Bertin Pharma, formerly Spi-Bio). Briefly, brain slides were pretreated with high-pressure autoclaving (2.1 x 10^5 Pa) for 30 min in citric acid (10 mM), pH 6.0, at 121°C, followed by treatment with 98% formic acid for 30 min and 4 M guanidine thiocyanate for 2 h at room temperature. Astrogliosis was evaluated by immunostaining of glial fibrillary acidic protein using an anti-GFAP antibody (0.5 mg/ml diluted 1:1,000; BD Biosciences) after hydrated autoclaving for epitope exposure. Immunohistochemical detection was achieved with biotinylated secondary antibodies according to the manufacturer's instructions (ARK animal research kit; Dako). Tissue sections were scanned with a NanoZoomer 2.0RS scanner (Hamamatsu Photonics) and analyzed using NanoZoomer digital pathology software (Hamamatsu Photonics).

3.3.4. Immunoblot analysis.

Brain tissues from tg mice were homogenized to 10% (wt/vol) in sterile water using disposable syringes and needles of decreasing diameters (18 gauge to 21 gauge), aliquoted, and stored at -80°C. The brain homogenate protein content was determined using a micro-bicinchoninic acid assay kit (Life Technologies). For the proteinase digestion reactions, 50 to 70 µg total protein (final sample protein concentration, 1 to 1.4 mg/ml) was treated with 150 µg/ml of proteinase K (Life Technologies) for 45 min at 37°C. Reactions were terminated by boiling the samples in 2.5X Laemmli buffer (150 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 25% glycerol, 5% [wt/vol] SDS, 12.5% ß-mercaptoethanol) at 95°C for 10 min. Samples (10 to 15 µg) were resolved on 12% NuPAGE bis-Tris gels (Life Technologies) and transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore). The membranes were blocked for 1 h at room temperature

with 5% (wt/vol) non fat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20

(TBST). Detection was performed using primary monoclonal antibody BAR224 (0.2 µg/ml

diluted 1:10,000 in 5% [wt/vol] nonfat dry milk in TBST; Bertin Pharma) or 8G8 (0.2 µg/ml

diluted 1:2,000; Bertin Pharma), secondary horseradish peroxidase-conjugated goat anti-mouse

IgG antibody, and chemiluminescent substrate (diluted 1:10,000; Life Technologies). Images were

acquired on X-ray film (Super Rx; Fujifilm). PK-res PrP^{CWD} glycoform ratios were determined

using three animals per inoculum group; samples were resolved by Western blotting and detected

with X-ray film. Quantification of PK-res PrP^{CWD} ratios was performed using ImageJ soft-ware

(NIH). Independent triplicate measurements from each sample group were averaged, and the

values were compared using GraphPad Prism (version 5.04) software.

3.4. Results

3.4.1 Transmission of experimental CWD into tg-deer-PRNP mice.

To evaluate the transmission properties of CWD prions derived from experimentally infected

white-tailed deer of different *PRNP* genotypes (Johnson et al., 2011), tg33 mice (expressing deer

wt-PrP^C) or tg60 mice (expressing deer S96-PrP^C) (Meade-White et al., 2007; Race et al., 2011)

were inoculated intracerebrally with deer CWD brain homogenates of 10% or 1% (wt/vol). All

CWD inocula (wt/wt, S96/wt, H95/wt, H95/S96) resulted in clinical prion disease in mice

expressing deer wt-PrP^C (tg33$^{-/+}$ and tg33$^{+/+}$ mice) (Fig. 3.1 and Table 3.1). Mice presented with

similar disease signs, including hyperactivity, kyphosis, ataxia, and myoclonus. Clinical signs

variably progressed into a general weakening, at which time the animals were euthanized. tg33

mice inoculated with the H95/S96 CWD agent had significantly longer incubation periods than

mice receiving the other CWD inocula (the Kruskal-Wallis test with Dunn's multiple-comparison posttest P<0.05) (Fig. 3.1 A, C, and D). No significant differences in the incubation periods were observed between tg33 mice inoculated with the S96/wt, H95/wt, or wt/wt CWD inoculum (the Kruskal-Wallis test with Dunn's multiple-comparison posttest, P < 0.05) (Fig. 3.1).

Figure 3.1. Transmission of CWD allotypes into transgenic mice expressing deer wt or S96-PrP^C. (A) Susceptibility of tg33 (wt-PrP^C) mice to infection with 10% (wt/vol) Bh from deer with CWD. (B) S96-PrP^C (tg60) mice developed clinical prion disease only when inoculated with CWD prions derived from deer expressing H95-PrP^C. Mice inoculated with wt/wt (open circles) or S96/wt (open squares) CWD prions did not show clinical signs. Symbols with crosses represent animals euthanized due to intercurrent disease. (C and D) Comparison of incubation periods in tg33$^{+/+}$ and tg33$^{+/-}$ mice. *** Significant differences between groups (the Kruskal-Wallis test with Dunn's multiple-comparison posttest, $P < 0.05$). The Mann-Whitney test ($P < 0.05$) was used to compare the distribution of incubation periods in tg60 mice.
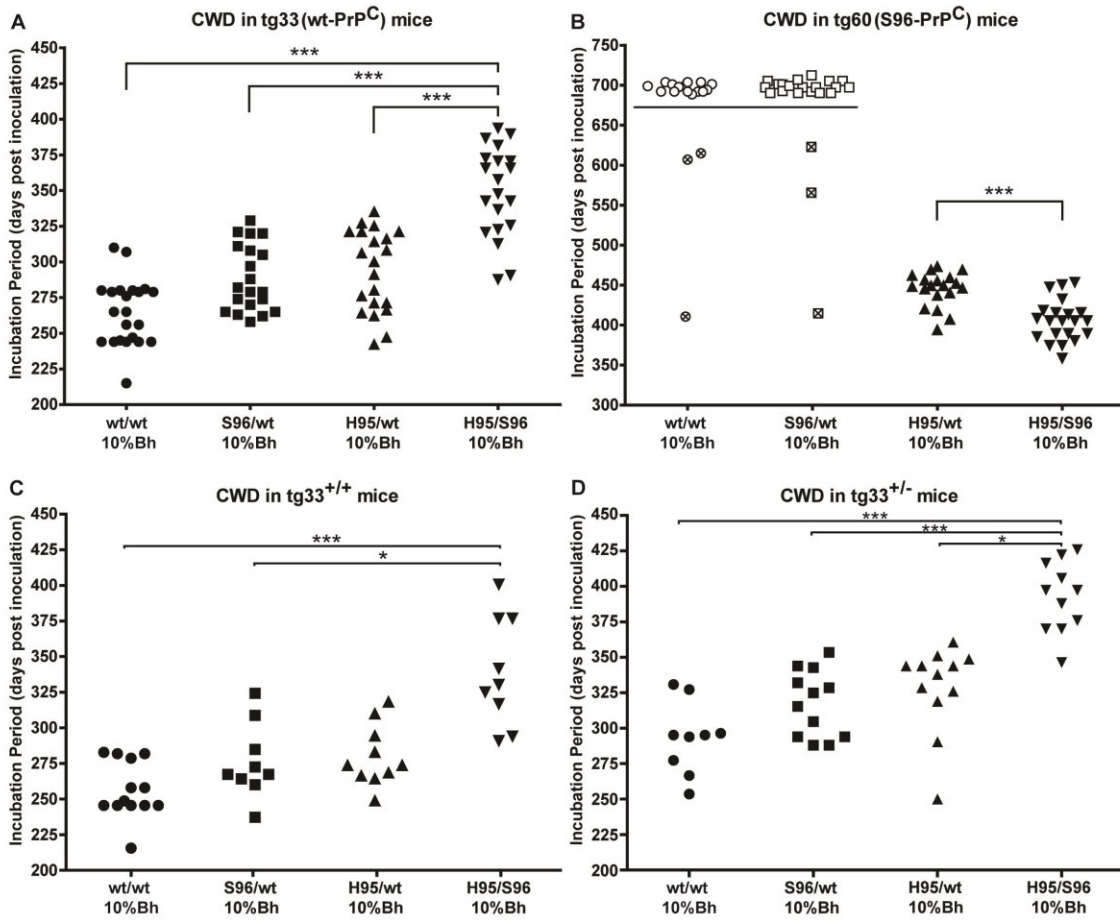
Table 3.1. Summary of transmission experiments and prion disease phenotypes of tg-deer-*PRNP* mice inoculated with white-tailed deer CWD.

Inocula	tg-deer -PRNP	CWD(bh) Dose %	Positive[a] /total	Incubation Period- Range (dpi)	PrP-Res type	PrP^CWD distribution pattern
Deer CWD-wt/wt	tg33	10%	22/22	215 - 310 dpi	High MW	Widespread "W"
	tg33	1%	8/8	256 - 345 dpi	High MW	Widespread "W"
	tg60	10%	0/18	> 700 dpi	Negative	Not Determined
	tg60	1%	0/10	> 600 dpi	Negative	Not Determined
Deer CWD-S96/wt	tg33	10%	20/20	258 - 329 dpi	High MW	Widespread "W"
	tg33	1%	8/8	225 - 357 dpi	High MW	Widespread "W"
	tg60	10%	0/21	> 700 dpi	Negative	Not Determined
	tg60	1%	0/10	> 600 dpi	Negative	Not Determined
Deer CWD-H95/wt	tg33	10%	21/21	242 - 335 dpi	High MW	Widespread "W"
	tg33	1%	10/10	316 - 356 dpi	High MW	Widespread "W"
	tg60	10%	19/19	394 - 473 dpi	Low MW	Localized "L"
	tg60	1%	10/10	465 - 608 dpi	Low MW	Localized "L"
Deer CWD-H95/S96	tg33	10%	20/20	288 - 394 dpi	High MW	Widespread "W"
	tg33	1%	9/9	323 - 433 dpi	High MW	Widespread "W"
	tg60	10%	21/21	359 - 454 dpi	Low MW	Localized "L"
	tg60	1%	16/16	359 - 554 dpi	Low MW	Localized "L"
tg60CWD -H95/wt	tg33	10%	15/15	340 - 383 dpi	High MW	Widespread "W"
	tg60	10%	16/16	310 - 380 dpi	Low MW	Localized "L"
tg60CWD -H95/S96	tg33	10%	7/7	373 - 409 dpi	High or Low	"L" or "W"
	tg33	1%	8/8	397 - 448 dpi	High or Low	"L" or "W"
	tg33	0.0001	8/8	329 - 490 dpi	High or Low	"L" or "W"
	tg60	10%	18/18	331 - 369 dpi	Low MW	Localized "L"
Un-infected Deer wt/wt	tg33	10%	0/6	> 560 dpi	Negative	Negative
	tg60	10%	0/5	> 560 dpi	Negative	Negative

In contrast to the susceptibility of the tg33 mouse line, mice expressing deer S96-PrP^C (tg60) developed clinical disease only when inoculated with CWD agents derived from deer expressing the *H95-PRNP* allele (Fig. 3.1 B). Mice inoculated with the H95/S96 and H95/wt CWD allotypes were clinically positive for prion disease between 359 and 473 dpi. Affected mice became lethargic with myoclonus, kyphosis, labored breathing, and ataxic gait characterized by limb weakness. Incubation periods were significantly different between tg60 mice inoculated with the H95/S96 CWD agent and tg60 mice challenged with the H95/wt CWD agent (Mann-Whitney test, $P < 0.05$). S96-PrP^C mice inoculated with the wt/wt and S96/wt CWD agents did not develop prion disease at >700 days postinoculation.

3.4.2. Neuropathology of *tg-deer-PRNP* mice infected with CWD agents.

To define the neuropathological hallmarks and assess differences between groups of mice inoculated with the different CWD inocula, sagittal and coronal brain sections were examined histologically for spongiform changes and immunohistochemically for PrP^{CWD} aggregates and GFAP-positive astroglia (Fig. 3.2). CWD-infected tg33 (wt-PrP^C) mice presented with extensive pathology in various brain regions and were characterized by neuronal loss, spongiform change, the widespread accumulation of PrP^{CWD} aggregates, and astrogliosis (Fig. 3.2 A to H). The distribution of pathological changes in the brain (i.e., PrP^{CWD} distribution) was similar between mice inoculated with the four CWD inocula (Fig. 3.3 A to G). The average spongiform change scores of various brain structures were similar among the infected tg33 mice (Fig. 3.2 Q). In general, the lesions (vacuolation and PrP^{CWD} accumulation) observed in the forebrain and

cerebellum agrees with previous results obtained with this transgenic mouse line after infection with CWD prions from other sources (Meade-White et al., 2007). Additionally, the granular layer of the cerebellum had areas of neuronal loss, where dense PrP^{CWD} aggregates surrounded by GFAP-positive astrocytes were revealed in consecutive tissue sections (Fig. 3.2 F to G). The spongiform changes and cell death in the cerebellum of tg33 mice were less conspicuous in the molecular layer and more abundant in the white matter and the Purkinje cell layers (Fig. 3.2 H). Infection of tg33 mice resulted in more prominent PrP^{CWD} accumulation in the corpus callosum (Fig. 3.2 A and B) than that described in other studies (Meade-White et al., 2007; Race et al., 2011).

Figure 3.2. Neuropathology of *tg-deer-PRNP* mice following the first passage of white-tailed deer CWD allotypes. (A and B) Accumulation of wt-PrP^{CWD} aggregates in tg33 mice. The regional distribution of wt-PrP^{CWD} aggregates was similar in mice receiving different CWD inocula (Fig. 3.3 A to G). (C to E) Hippocampal degeneration (box in panel A) was characterized by spongiform change and a loss of pyramidal neurons of the Ammon's horn (C1 and C3), accompanied by the extensive accumulation of PrP^{CWD} aggregates and abundant astrocytosis (GFAP). (F and G) Cerebellum pathology involved the loss of granular neurons and the presence of prion protein deposits flanked by astrocytes (as seen in sequential tissue sections). (H) Vacuolation was observed in Purkinje neurons and cerebellar white matter. (I and J) Detection of S96-PrP^{CWD} aggregates in tg60 mice infected with the H95/wt and H95/S96 CWD allotypes. The distribution of PrP^{CWD} aggregates was similar between animals receiving the H95$^+$ deer CWD agent (Fig. 3.3 J to K). (K) S96-PrP^{CWD} aggregates in the hippocampus were noticeable at a higher magnification of the small box in panel J. (L and M) Abnormal prion protein deposits and spongiosis in thalamic nuclei shown by a higher magnification of the large box in panel J. (N to P) Cerebellar pathology included white matter vacuolation (N) and astrocytosis (O) that colocalized with diffuse and punctate protein aggregates (P). (Q) Lesion profile of tg33 mice infected with deer CWD allotypes. (R) Lesion profile of tg60 mice infected with the H95$^+$ deer CWD agent. Brain regions are as follows: 1, medulla; 2, cerebellum; 3, superior colliculus; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septum; 8, posterior cortex; 9, anterior cortex. Bars, 2.5 mm (A and I), 1 mm (B and J), 850 μm (E), 300 μm (C, O and P), 125 μm (D, L and H), and 60 μm (F, G, K, M, and N). PrPCWD detection was achieved with anti-PrP monoclonal antibody BAR224. (A) Brain section from a tg33 mouse infected with S96/wt CWD prions at 270 dpi; (B) brain section

from a tg33 mouse infected with H95/S96 CWD prions at 387 dpi, (I) brain section from a tg60 mouse infected with H95/S96CWDprions at 375 dpi; (J) brain section from a tg60 mouse infected with H95/S96 CWD prions at 414 dpi.
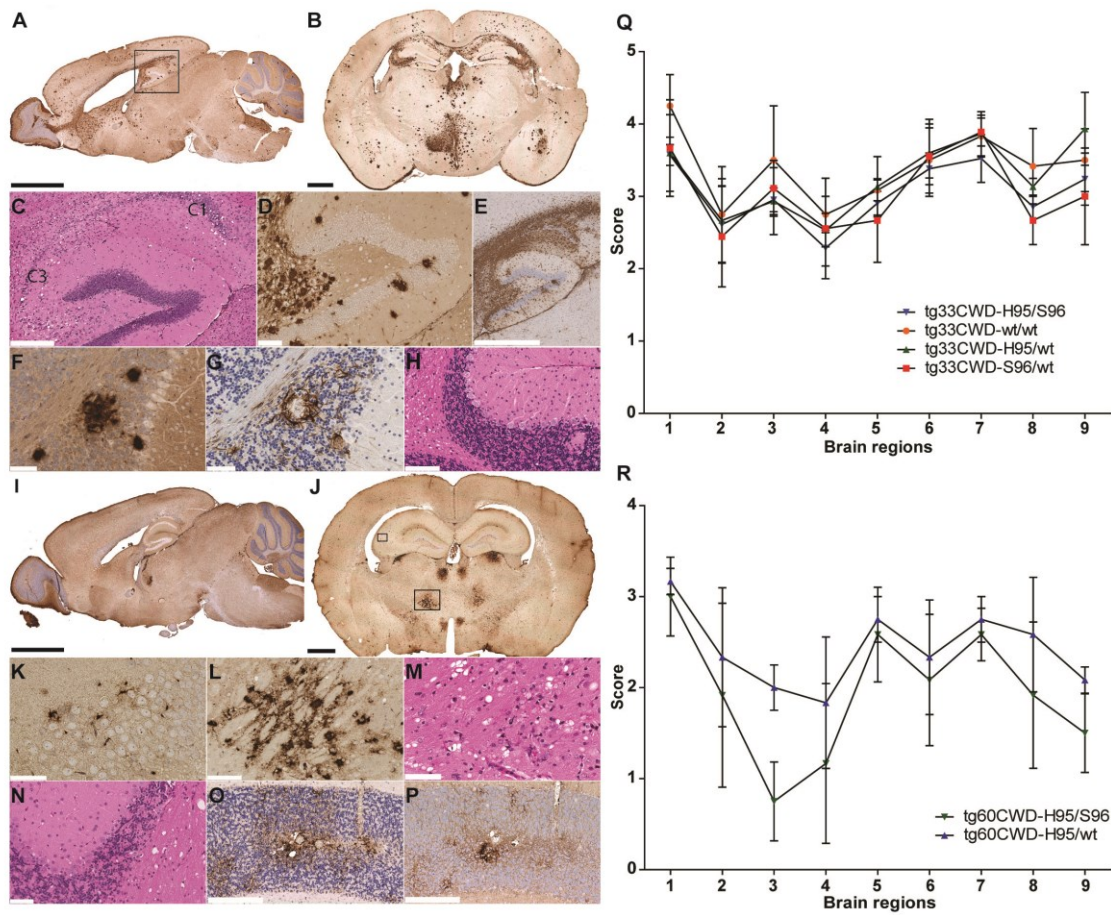
Figure 3.3. Distribution of PrP^{CWD} aggregates in the brains of *tg-deer-PRNP* mice inoculated with different white-tailed deer CWD allotypes. (A to G) PrP^{CWD} aggregates in the brains of tg33 mice inoculated with different white-tailed deer CWD allotypes. (H and I) Abnormal PrP aggregates were detected after 700 dpi in the brains of tg60 mice without clinical signs inoculated with the wt/wt or S96/wt CWD allotypes. (J and K) Only tg60 mice inoculated with H95$^+$ CWD allotypes had clinical signs and were consistently positive for PrP^{CWD} aggregates. (K) Coronal brain sections from a clinically ill tg60 mouse infected with H95$^+$ CWD prions (414 dpi). (L and M) Brain sections of mock-infected tg33 and tg60 mice. Bars, 1mm(A to D, J, and K), 2.5mm(E to I and M), and 5mm (L). Tissue sections were stained with anti-PrP monoclonal antibody BAR224.

tg33(wt-PrP^C) mice

Inocula:

wt/wt CWD	H95/S96 CWD	H95/wt CWD	S96/wt CWD

A 327 dpi | B 406 dpi | C 314 dpi | D 321 dpi

E 280 dpi | F 288 dpi | G 271 dpi

wt/wt CWD | H95/S96 CWD | H95/wt CWD

tg60(S96-PrP^C) mice

wt/wt CWD | S96/wt CWD

H 726 dpi | I 662 dpi

H95/S96 CWD

K

H95/wt CWD

J 473 dpi

Mock-inoculated tg33 | Mock-inoculated tg60

L | M

111
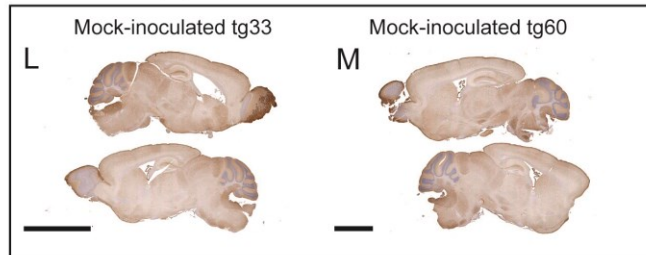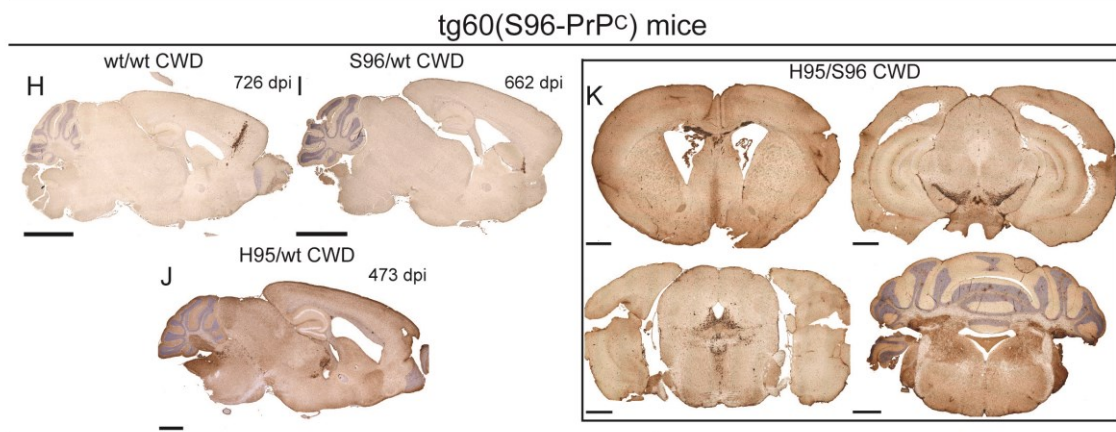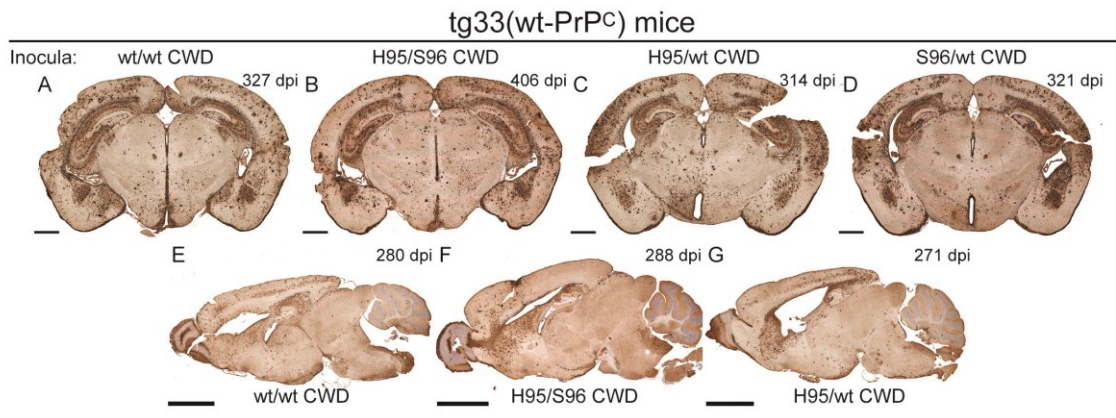
The susceptibility of S96-PrPC (tg60) mice to CWD infection was strongly influenced by the invading CWD allotype. All tg60 mice exposed to the H95/wt or H95/S96 CWD agent developed clinical prion disease with similar neuropathologies (Fig. 3.2 I to P and R and 3.3 J to K). The distribution and severity of the neuropathological changes observed in diseased tg60 mice infected with H95[+] CWD followed a consistent lesion pattern (Fig. 3.2 I to P and R and 3.3 J to K). Spongiform degeneration and abnormal S96-PrP[CWD] aggregates were localized in the caudoputamen and the corpus callosum and extended down the septum to the diagonal band nucleus (Fig. 3.3 K). Both vacuolation and PrP[CWD] deposition were of milder intensity in the cerebral cortex and hippocampus than in the other brain areas; however, immunohistochemical staining revealed the presence of small, punctate S96-PrP[CWD] aggregates at higher magnification (Fig. 3.2 K). Pathological changes were more severe in various regions of the thalamus, including the medial-dorsal, ventral-medial, and ventral anterior-lateral thalamic nuclei (Figure. 3.2 I to J, L, and M and 3.3 J) and also involved the zona incerta, cerebral peduncle, and subthalamic and hypothalamic nuclei (Fig. 3.2 I to J and 3.3 J). In the midbrain, lesions were localized in the substantia nigra adjacent to the ventral tegmental area and extended to periaqueductal gray and adjacent structures, including the raphe nucleus, mesencephalic reticular formation, and superior cerebellar peduncle (Fig. 3.2 I and 3.3 K). Pathology was also observed in the hindbrain and affected various regions, including the median raphe nucleus and pontine reticular nucleus (Fig. 3.3 K). In the cerebellum, the spongiform change was the most prominent in the white matter; however, small vacuoles were also observed in the molecular, Purkinje, and granular layers, with the granular layer showing loss of granular neurons (Fig. 3.2 N). PrP[CWD] staining revealed either diffuse deposits (lightly stained) or larger confluent aggregates in the cerebellar nuclei and the

granular layer (Fig. 2 I and P and 3 K).

A few tg60 (S96-PrPC) mice that did not have clinical signs and that were challenged with the wt/wt or S96/wt CWD agents (3/28 and 3/31 mice, respectively) had detectable prion aggregates at 700 days postinoculation (Fig. 3 H to I), highlighting the low efficacy of these CWD agents for establishing infection in this transgenic line. The accumulation of PrPC^{WD} aggregates in these particular mice did not follow the PrPCWD distribution patterns described in the other mice.
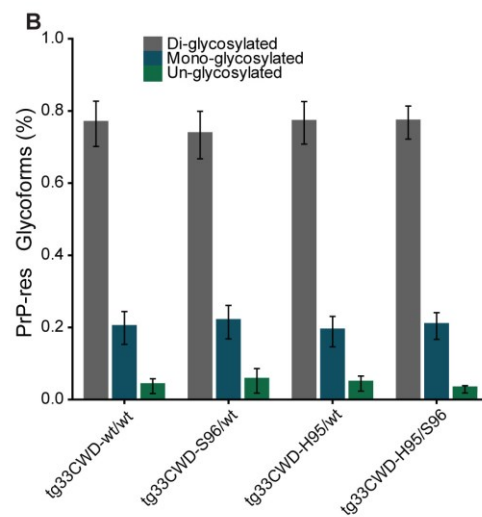

3.4.3. PK-res PrP^{CWD} glycotypes in transgenic mice expressing deer PrP^{C}.

Distinct PK-res PrP^{CWD} isoforms have been associated with different prion strains (Bruce and Dickinson, 1987; Parchi et al., 1997; Hill et al., 2003). PK-res PrP^{CWD} can vary in their molecular masses, glycoform ratios, and other biochemical properties related to the structural stability of the abnormal PrP conformers (Safar et al., 1998). These properties have been interpreted to be conformational differences in the structures of the misfolded PrP molecules that carry the information that defines different prion strains (Bruce and Dickinson, 1987; Parchi et al., 1997; Hill et al., 2003; Safar et al., 1998). To compare the PK-res PrP^{CWD} in mice infected with the different CWD inocula, brain homogenates were digested with proteinase K and analyzed by Western blotting using anti-PrP monoclonal antibodies 8G8 (which recognizes deer PrP amino acid residues 100 to 105) or Bar224 (which epitope comprises deer PrP residues 144 to 154). All clinically affected tg33 mice were PK-res PrP^{CWD} positive; no differences with respect to molecular masses and glycoform patterns were observed (Fig. 3.4). Although the electrophoretic profile of PK-res PrP^{CWD} from clinically affected tg60 mice was similar between mice inoculated with the H95/wt or H95/S96 CWD allotypes, this PK-res PrP^{CWD} type was distinct from that

observed in tg33 mice. The gel migration of the proteinase K cleavage products indicated that

S96-PK-res PrP^{CWD} has a lower molecular mass than wt-PK-res PrP^{CWD} (Fig. 3.4). PK-res PrP^{CWD}

was not detected at 700 dpi in brain homogenates from tg60 mice inoculated with the wt/wt or

S96/wt CWD allotypes.

Figure 3.4. PK-res PrP^{CWD} in *tg-deer-PRNP* mice inoculated with different CWD allotypes.

(A) PK-res PrP^{CWD} from brains of prion-affected tg33 (wt-PrP^C) and tg60 (S96-PrP^C) mice. Brain homogenates were digested with proteinase K (PK) and analyzed by SDS-PAGE and Western blotting. Lanes M, molecular size markers. PrP-res from tg33 mice had similar molecular masses after enzymatic cleavage (A) and equivalent glycoform ratios (B). S96 PK-res PrP^{CWD} has a lower molecular mass and was detectable only in brain homogenates derived from tg60 mice infected with H95$^+$ CWD allotypes. UI, homogenates from tg mice inoculated with uninfected deer brain homogenate. PK-res PrP^{CWD} detection was achieved with anti-PrP monoclonal antibody BAR224.

A

MW

50 KDa
37 KDa

25 KDa
20 KDa

15 KDa

10 KDa

MW

MW

PK: - + + + + +   - + + + + +

tg-deer-*PRNP* line: tg33   tg60

Inoculum: UI UI wt/wt S96/wt H95/wt H95/S96   UI UI H95/wt H95/S96 wt/wt S96/wt

B

Di-glycosylated
Mono-glycosylated
Un-glycosylated

PrP-res Glycoforms (%)

1.0
0.8
0.6
0.4
0.2
0.0

tg33CWD-wt/wt   tg33CWD-S96/wt   tg33CWD-H95/wt   tg33CWD-H95/S96
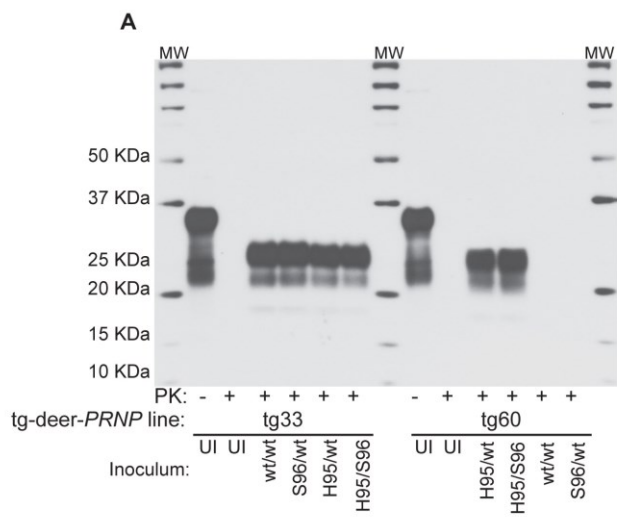
3.4.4. Serial transmission of passage 1 tg60CWD-H95$^+$ isolates into *tg-deer-PRNP* mice. To

evaluate the transmission properties of tg60 (S96-PrP^C) mouse-passaged CWD prions, we

inoculated these isolates into both the tg33 and tg60 mouse lines. Serial transmission of first-

passage tg60CWD-H95$^+$ isolates back into tg60 mice (syngeneic passage) resulted in a reduction

of the incubation periods (Fig. 3.5 A and Table 3.1). The disease signs, biochemical PK-res

PrP^{CWD} glycotype, and neuropathology resembled those after first passage (Fig. 3.5 B to D).

Passage of tg60CWD-H95$^+$ isolates into tg33 mice (allogeneic passage) resulted in two different

prion disease presentations. After exposure to 10% (wt/vol) tg60CWD-H95/wt brain homogenate,

the mice had extended incubation periods compared to those of tg33 mice infected with the

H95/wt deer CWD allotype (Fig. 3.6 A and Table 3.1). Disease signs and pathological hallmarks

were similar to those described during the first passage of deer CWD prions in tg33 mice,

characterized by hyperactivity, a widespread distribution of aggregates in the brain, and high-

molecular-mass PK-res PrP^{CWD} (Fig. 3.6 B and C). Transmission of the tg60CWDH95/S96 isolate

into tg33 mice resulted in divergent prion disease phenotypes. Inoculation of 10% brain

homogenates resulted in extended incubation periods, with some mice developing disease signs

and pathology characteristic of tg33 mice infected with the deer CWD agents, while others

developed disease signs and neuropathology that resembled the disease phenotype described for

tg60 mice (Fig. 3.6). Evaluation of proteinase K-resistant PrP in brain homogenates from affected

mice revealed PK-res PrP^{CWD} glycotypes of distinct molecular masses (Fig. 3.6 B). Passage of 1%

and 0.0001% (wt/vol) brain homogenates resulted in further extension of the incubation period

and increased the abundance of mice presenting with lethargy (like tg60 mice), accompanied by the accumulation of low-molecular-mass PK-res PrP^{CWD} and localized deposition of PrP aggregates in brain (Fig. 3.6).

Figure 3.5. Serial passage of tg60 (S96-PrP^C)-passaged CWD prions. (A) Syngeneic transmission of tg60CWD-H95$^+$ isolates into tg60 mice led to reduction in the incubation period following intracerebral inoculation of 10% (wt/vol) brain homogenates. (B) S96 PK-res PrP^{CWD} properties were maintained following secondary passage in tg60 mice. S96 PK-res PrP^{CWD} has a lower molecular mass than wt PK-res PrP^{CWD} derived from tg33 mice. Lanes M, molecular size markers. (C and D) Distribution of S96-PrP^{CWD} aggregates in the brains of tg60 mice infected with tg60CWD-H95$^+$ prions. Immunohistochemical comparison revealed a similar distribution of abnormal PrP^{CWD} aggregates, as observed in tg60 mice from the first passage of H95$^+$ deer CWD agent (Fig. 3.2 I to J and 3.3 J and K). Detection of abnormal PrP (B to D) was performed with antibody BAR224. Bars, 2.5 mm.

A tg60CWD-H95⁺ Syngeneic passage

B

PK: - + - + + + + + + +
tg-deer-*PRNP*: 33 33 60 60 | 60 60 60 60 | 33 33
UI | Deer-H95/wt Deer-H95/S96 H95/wt H95/S96 | Deer-H95/wt Deer-H95/S96
Inocula: | P2 tg60 CWD |

C

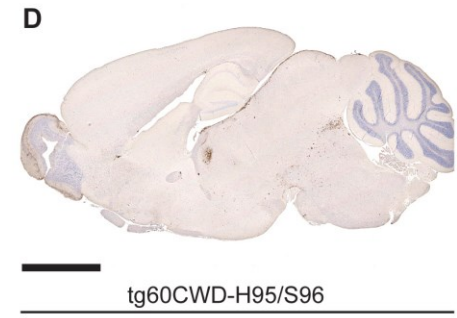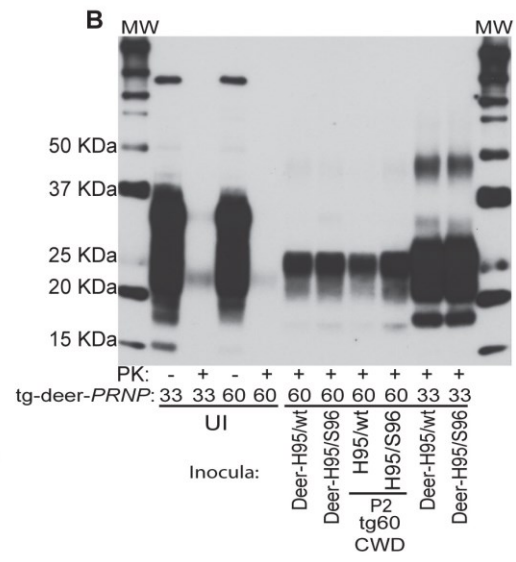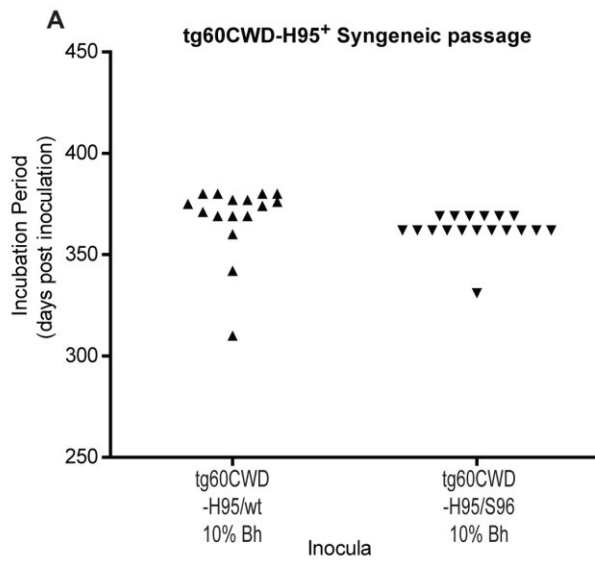Inocula: tg60CWD-H95/wt

D

tg60CWD-H95/S96

Figure 3.6. Allogeneic transmission of tg60 (S96-PrP^C) mouse-passaged CWD prions into tg33 mice. (A) Incubation periods of tg33 mice upon challenge with passage 1 tg60CWD-H95$^+$ isolates. Passage of tg60CWD-H95/S96 brain homogenates gave rise to different clinical presentations (hyperactivity versus lethargy) resembling the disease phenotypes described for both *tg-deer-PRNP* lines during the first passage of deer CWD prions. Black symbols, tg33 animals with hyperactive disease presentation, high-molecular-mass PrP-res, and a widespread distribution of brain PrP^{CWD} aggregates; orange symbols, tg33 mice with a lethargic presentation, low-molecular-mass PrP-res, and a localized distribution of PrP^{CWD} aggregates. (B) PrP-res glycotypes in brains of tg33 mice inoculated with different tg60CWD-H95$^+$ isolates. Infected tg33 mice accumulated different proteinase K-resistant PrP types resembling those observed after the first passage of deer CWD prions. Lane M, molecular size markers. (C) Divergent histological phenotypes in tg33 mice infected with tg60CWD-H95/S96 or tg60CWD-H95/wt brain homogenates. Bars, 2.5 mm. Detection of abnormal PrP was performed with anti-PrP monoclonal BAR224.

A

tg60CWD-H95⁺ allogeneic passage

Incubation Period (days post inoculation)

Inocula

Inocula (x-axis labels):
tg60CWD-H95/wt 10% Bh
tg60CWD-H95/S96 10% Bh
tg60CWD-H95/S96 1% Bh
tg60CWD-H95/S96 10⁻⁴ Bh

B

tg33(wt-PrPᶜ)

MW

37 KDa
25 KDa
20 KDa
15 KDa

PK: + + + + + + + + + + + + +

Inocula: tg60CWD -H95/S96 10% Bh | tg60CWD -H95/S96 1% Bh | tg60CWD -H95/S96 10⁻⁴ Bh | Deer-H95/wt | Deer-H95/S96 | tg60CWD -H95/wt 10% Bh

C

409dpi   445dpi   383dpi

Inocula: tg60CWD-H95/S96 10% Bh   tg60CWD-H95/S96 1% Bh   tg60CWD-H95/wt 10% Bh

3.5 Discussion

To explore the transmission properties of CWD prions derived from white-tailed deer of four different *PRNP* genotypes (Johnson et al., 2011), we inoculated transgenic mice expressing deer prion proteins associated with susceptibility (tg33 mice expressing deer wt-PrP^C) or resistance (tg60 mice expressing deer S96-PrP^C) to CWD prions (Meade-White et al., 2007; Race et al., 2011). Transmission of the deer H95/wt and H95/S96 CWD allotypes resulted in the emergence of a distinct CWD strain (H95$^+$). This novel prion agent was identified when brain homogenates from deer containing H95-PrP molecules were transmitted into tg60 mice. Passage of these deer brain homogenates into tg33 mice, however, resulted in a prion disease phenotype indistinguishable from that observed following infection with the wt/wt or S96/wt CWD agents. The ability of H95$^+$ CWD agent to cause clinical prion disease in tg60 mice, which have been shown to be resistant to other CWD isolates, indicates that a new strain has emerged (Jonson et al., 2011; Meade-White et al., 2007; Race et al., 2011). Our data show that the passage of CWD (wt/wt pool) through deer with the H95/wt and H95/S96 allotypes resulted in a mixture of at least two CWD strains, distinguishable on the basis of the *tg-deer-PRNP* genotype in which they were propagated.

Upon first passage into tg33 mice, all deer CWD agents resulted in similar disease signs, PK-res PrP^{CWD} glycotypes, and neuropathological features, suggesting that expression of wt-PrP^C favored the propagation of a CWD strain (prion conformer) common to all inocula. We refer to this agent as "Wisc-1". Our results suggest that Wisc-1 is similar to strain CWD-1described by Angers et al. (2010). The white-tailed deer sample analyzed in the study of Angers et al. (2010) was a wt/wt

CWD isolate from Wisconsin. Whether Wisc-1 and CWD-1 are identical is difficult to ascertain, as the white-tailed deer agents were passaged in different transgenic mice. The H95$^+$ CWD strain differs from the Wisc-1, CWD-1, and CWD-2 strains (Angers et al., 2010; 2014).

We found that inoculation of the H95/S96 CWD agent into tg33 mice resulted in incubation periods significantly different from those obtained by inoculation of CWD prions of the other allotypes. The absence of wt-PrP^{CWD} in this inoculum and, thus, the lack of homologous prion conversion likely contributed to the prolonged incubation period. The presence of more than one prion conformer within this inoculum may result in competition between agents, leading to propagation interference and extension

of the incubation periods (Bartz et al., 2000; Bartz et al., 2004; Bartz et al., 2007; Shikiya et al., 2010).

Incubation periods were not significantly different between tg33 mice infected with the wt/wt, H95/wt or S96/wt CWD agents. Additionally, all tg33 mice presented the same prion disease phenotype irrespective of the CWD inoculum that they received. One possible interpretation for the phenotypic similarities observed between tg33 mice is that the Wisc-1 conformers have an adaptive advantage in hosts (either in deer or in tg mice) expressing wt-PrP^C. The differences in incubation periods between the H95/wt CWD allotype- and H95/S96 CWD allotype-infected tg33 mice suggest that the PrP^C sequence in these deer impacted the proportion of accumulated CWD strains. It has previously been demonstrated in hamster coinfection experiments that the ratio of the strains in a prion mixture influences the emergence of the fastestreplicating or dominant strain

(Bartz et al., 2000; Bartz et al., 2004; Shikiya et al., 2010).

The differential susceptibility to prion infection is modulated by PrP^C amino acid sequence variability and the invading prion strain (Dickinson et al., 1968a; Bruce et al., 1991;Mead et al., 2008;Brown et al., 2012;Prusiner et al., 1990; Lee et al., 2001; Westaway et al., 1987). Both natural and experimental infections support the association of S96-PrP^C with reduced susceptibility and the slower progression of CWD (Thomsen et al., 2012, Johnson et al., 2003; O'Rourke et al., 2004; Johnson et al., 2006a; Johnson et al., 2011; Miller et al., 2012; Meade-White et al., 2007; Race et al., 2011; Angers et al., 2014). Tg60 (S96-PrP^C) mice were previously shown to be resistant to CWD isolates from different cervid species (Meade-White et al., 2007; Race et al., 2011). In our study, tg60 mice inoculated with the wt/wt or S96/wt CWD agents did not present with clinical disease after >700 dpi; however, mice receiving the H95/wt and H95/S96 CWD allotypes developed disease signs and presented consistent neuropathology and PK-res PrP^{CWD} glycotypes. A second passage of the tg60CWD-H95$^+$ isolates into tg60 mice resulted in a reduction of the incubation periods and similar phenotypic characteristics.

Allogeneic transmission of the first-passage tg60CWD-H95$^+$ isolates into tg33 mice resulted in the development of prion disease with two distinct phenotypes resembling those caused by the Wisc-1 and H95$^+$ prion strains. While some animals presented with hyperactivity and displayed a widespread accumulation of disease-associated PrP in the brain as well as high-molecular-mass PK-res PrP^{CWD}, others were lethargic with localized PrPCWD deposits and a distinct PK-res PrP^{CWD} glycotype. Transmission of a diluted tg60CWDH95$^+$ inoculum resulted in more mice presenting the tg60-like phenotype. This suggests that the tg60 donor mouse, which preferentially

amplified the H95[+] strain, contained a persistent Wisc-1 fraction that was amplified upon passage

at a high dose (10% Bh) in tg33 mice. Transmission of lower doses of the inoculum likely altered

the proportion of the two prion conformers, favoring the propagation of the H95[+] strain. A similar

outcome was observed when dilutions of the transmissible mink encephalopathy agent were

passaged in hamsters, resulting in the isolation of the Hyper and Drowsy strains (Bartz et al.,

2000). Transmission of tg60CWDH95[+] isolates into tg33 mice indicates that individual tg60 mice

accumulated mixtures of CWD agents. Although prion transmission experiments in tg mice do not

always recapitulate what is observed in the wild (i.e. tg60 mice are resistant to a number of

different CWD strains, whereas 96S homozygous deer are naturally infected), natural scrapie and

CWD isolates have been shown to contain strain mixtures that can be differentiated by serial

passage in mouse models or by histopathological and biochemical analyses (Angers et al.,

2014;Thackray et al., 2011; 2012; Mazza et al., 2010).

Deer with *S96-PRNP* alleles can be infected with the CWD agent but have extended preclinical

periods, suggesting that they could be infectious over longer periods of time than wt homozygous

deer (Johnson et al., 2011; Miller et al., 2012; Race et al., 2011). Additionally, in areas where

CWD is endemic, white-tailed deer with *S96-PRNP* alleles likely have a fitness advantage over

deer with the more susceptible genotypes, and as a result, the resistance allele may become more

abundant in the population (Robinson et al., 2012a). An increase in the *S96-PRNP* allele

frequency could also affect the potential for the selection of CWD strains able to infect deer with

resistant genotypes. Likewise, other *PRNP* alleles associated with extension of the CWD

preclinical phase, such as *H95-PRNP*, could also be subjected to a disease-driven increase in

white-tailed deer populations. Our transmission data show that deer expressing H95-PrP[C]

accumulate a CWD strain capable of infecting deer with *S96-PRNP* genotypes, unlike other CWD agents. An increase in the frequency of *H95-PRNP* would also increase the likelihood of the emergence of H95$^+$ CWD prions. Our data suggest that white-tailed deer expressing different PrP^C allotypes can accumulate and transmit CWD strain mixtures. CWD epizootics involve multiple factors, including the contagious nature of the agent, host-pathogen interactions, agent strains, and cervid population genetics. Our data indicate that CWD strain emergence is modulated by amino acid polymorphisms in the cervid PrP. CWD transmission between hosts with different *PRNP* genotypes (Robinson et al., 2012b) has the potential to generate and select novel prion conformations. Deer expressing H95-PrP^C accumulate CWD prions with different transmission properties, as exemplified by its ability to infect resistant *S96-PRNP* mice. Finally, our study highlights the importance of characterizing the diversity of CWD strains and their potential for interspecies transmission, as various mammalian species are susceptible to experimental CWD infection (Hamir et al., 2006a; 2006b; Heisey et al., 2010; Mathiason et al., 2013). Although several lines of evidence suggest that humans are resistant to CWD prions (Kong et al., 2005; Marsh et al., 2005; Race et al., 2009; Race et al., 2014), not all CWD strains have been tested for their zoonotic potential. Our results demonstrating that H95$^+$ deer CWD prions have transmission properties different from those of CWD prions composed of wt-PrP or S96-PrP suggest the need for evaluation of the transmissibility of CWD allotypes.

CHAPTER 4.

EMERGENT STRAINS EXPAND THE HOST RANGE OF CHRONIC WASTING

DISEASE

This chapter was submitted for publication in The Journal Emerging Infectious Diseases and is reproduced here with additional data:

Herbst A*, Duque Velásquez C*, Triscott E, Aiken J & McKenzie D.

4.1 Abstract

Chronic Wasting Disease (CWD) is a cervid prion disease that is expanding geographically and increasing in prevalence. The ability of prions to infect other species is determined by the replicative compatibility between the invading prion strain and the amino acid sequence of the host prion protein, manifesting as a transmission barrier in some prion-host combinations. Identification of an emergent CWD strain (H95$^+$) with distinct properties compelled us to examine the host range of this strain. We show that the H95$^+$ strain infects mice with 100% penetrance, overcoming any species barrier imposed by structural constraints in the mouse prion protein. Conversely, hamsters were more susceptible to the prevalent CWD strains. Humans and mice share structural topologies in the prion protein that confer resistance against prevalent CWD strains. The ability of H95$^+$ to cross the species barrier in mice suggests this strain may have enhanced zoonotic potential relative to prevalent strains.

4.2 Introduction

Chronic wasting disease (CWD) is a contagious cervid prion disease spreading globally. CWD is enzootic in multiple cervid species, including deer and elk with major foci of disease located in the Colorado/Wyoming and Wisconsin/Illinois in the United States and Alberta/Saskatchewan in Canada (Williams and Young, 1980; USGS, 2016). Commercial movement of asymptomatic prion-infected elk from Canada has resulted in CWD in captive elk, red deer and sika deer in Korea (Lee et al., 2013b). The geographic range of CWD has further expanded with the recent identification of CWD in wild reindeer and moose in Norway (Benestad et al., 2016).

Prion diseases result from the conformational transformation of the host-encoded cellular prion protein (PrP^C) into protease-resistant, detergent-insoluble, beta-sheet rich, amyloidogenic conformers termed prions (PrP^{CWD}). Within their conformation, prion strains encipher the information that directs the templated-misfolding and aggregation of PrP^C molecules into additional prions (Prusiner, 1991; Bessen and Marsh, 1992b; Safar et al., 1998). The faithful and stable epigenetic transmission between prions and PrP^C molecules, across cells, between individuals and within populations defines a prion strain (Bruce and Dickinson, 1979; Carlson et al., 1989). Prion strains are characterized by replicable disease phenotypes (incubation period, clinical presentation, PrP^{CWD} biochemical properties, host range) that are stable under standard passage conditions, including the host *PRNP* genotype (Bessen and Marsh, 1992a, 1992b; Kimberlin et al., 1987).

Although PrP has high sequence homology among mammals, the ability of particular prion strains to cause disease in different species is limited and associated with a barrier to transmission. This barrier is determined by the conformational compatibility between a given strain and the host PrP^C (Prusiner et al., 1990). Similarly, within a species, PrP^C amino acid variants affect susceptibility to prion disease by limiting the efficiency and fidelity of prion conversion; polymorphic PrP^C can be a poor substrate for the invading prion strain (Carlson et al., 1989; Hunter et al., 1994; Duque Velásquez et al., 2015; Asante et al., 2015). This reduced fidelity of conversion following inter-genotypic transmission can result in prion mutation and novel prion strain emergence (Bessen and Marsh, 1992a; Kimberlin and Walker, 1978). The ability of BSE prions to cross the species barrier and infect humans (Bruce et al., 1997; Hill et al., 1997) raises concerns for CWD, given

the direct and environmental interactions between deer and cattle, exposure of hunters to CWD contaminated viscera, and venison consumption.

Several notable polymorphisms in the gene encoding PrP^C, *PRNP*, have been identified that affect CWD susceptibility, reviewed by Robinson (2012b). In white-tailed deer, for example, two *PRNP* alleles code for amino acid polymorphism at codons 95 (Q to H) and 96 (G to S). These alleles result in differential susceptibility to CWD agents present within enzootic areas (Johnson et al., 2011; Miller et al., 2012). PrP amino acid polymorphisms at codons 132 (M to L) and 225 (S to F) also have susceptibility effects in elk and mule deer, respectively (Wolfe et al., 2014).

Evidence for the existence of multiple CWD prion strains is accumulating (Duque Velásquez et al., 2015; Bartz et al., 1998; Raymond et al., 2007; Angers et al., 2010; Perrot et al., 2012). Thus far, four CWD strains have been denoted (CWD1, CWD2, Wisc-1, H95$^+$). CWD1 and CWD2 were from cervids in the Colorado/Wyoming foci of infection (Angers et al., 2010) and likely reflects the circulation of prion agents between white-tailed deer, mule deer and elk. Wisc-1 is the prevalent strain in white-tailed deer from Wisconsin. The H95$^+$ CWD strain emerged from the modification/adaption of Wisc-1 in deer expressing the H95-PrP^C polymorphism (Duque Velásquez et al., 2015).

The transition from one host genotype to another impacts the biological properties of CWD strains. For example, direct transmission of mule deer CWD into hamsters did not result in clinical disease but if the CWD was first passaged through ferrets, an agent with tropism for hamsters emerged (Bartz et al., 1998). Hosts expressing the 96S PrP^C variant show resistance to CWD1,

CWD2, Wisc-1 and other undefined CWD strains (Duque Velásquez et al., 2015; Johnson et al., 2011; Miller et al., 2012; Angers et al., 2014) but can be more readily infected if exposed to CWD prions derived from deer expressing H95-PrP[C] (Duque Velásquez et al., 2015).

Secondary structure of host PrP influences the ability of a strain to propagate in a new host. Based on NMR studies, it has been hypothesized that amino acid variation at residues 165-175 (Figure 4.1) in PrP[C] results in a loop structure between alpha-helix 2 and beta-sheet 2 (α2-β2) whose flexibility influences the outcome of interspecies transmissions (Gossert et al., 2005; Gorfe et al., 2007; Sigurdson et al., 2009; Sigurdson et al., 2010; Bett et al., 2012; Kurt et al., 2014; Kurt et al., 2015). Mice and humans share a flexible loop sequence (SNQNN) whereas deer and elk possess a rigid loop (NNQNT). Multiple attempts to transmit CWD prions to mice have failed (Raymond et al., 2007; Bett et al., 2012; Kurt et al., 2014; Kurt et al., 2015; Browning et al., 2004; Meyerett et al., 2008; Trifilo et al., 2007). The transmission of prions occurs more readily when the loop structures match, e.g,. BSE transmits to humans and mice (Bruce et al., 1997, Hill et al., 1997). Experimental alteration of the mouse flexible loop to a rigid loop facilitated CWD transmission (Sigurdson et al., 2010; Kurt et al., 2015). As this loop structure is different between mice and cervids, we hypothesized that Wisc-1 and H95[+] would not be transmissible to mice. Loop structure similarity between hamsters (NNQNN) and cervids would predict transmission.

Figure 4.1. Alignment of the PrP^C primary structures from mammalian species expressing rigid or flexible α2-β2 loops. Cervids are characterized by rigid loops while mice, hamster and primates produce flexible loop topologies.

```
              10          20         30          40         50
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPP    Odocoileus virginianus wt
--MANLGYWLLALFVTMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP    Mus musculus
--MANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP    Homo sapiens
--MANLSYWLLALFVATWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP    Mesocricetus auratus
--MANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP    Macaca fascicularis
--MANLGCWMLVLFVATWSDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPP    Saimiri sciureus
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPP    Cervus canadensis M132


                            60          70         80          90
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-THS    Odocoileus virginianus wt
QGG--------TWGQPHGGGWGQPHGGSWGQPHGGSWGQPHGGG-WGQGGGGTHN    Mus musculus
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGGTHS    Homo sapiens
QGG-------GTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGGTHN    Mesocricetus auratus
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGGTHN    Macaca fascicularis
QGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-WGQGGGGTHN    Saimiri sciureus
QGG-------GGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-THS    Cervus canadensis M132


  100         110        120         130        140         150
QWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR    Odocoileus virginianus wt
QWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPMIHFGNDWEDRYYR    Mus musculus
QWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYVLGSAMSRPIIHFGSDYEDRYYR    Homo sapiens
QWNKPNKPKTSMKHMAGAAAAGAVVGGLGGYMLGSAMSRPMLHFGNDWEDRYYR    Mesocricetus auratus
QWHKPSKPKTSMKHMAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR    Macaca fascicularis
QWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR    Saimiri sciureus
QWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYR    Cervus canadensis M132


        160         170        180         190        200
ENMYRYPNQVYYRPVDQYNNQNTFVHDCVNITVKQHTVTTTTKGENFTETDIKM    Odocoileus virginianus wt
ENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKM    Mus musculus
ENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKM    Homo sapiens
ENMNRYPNQVYYRPVDQYNNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKM    Mesocricetus auratus
ENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKM    Macaca fascicularis
ENMYRYPSQVYYKPVDQYSNQNNFVHDCVNVTIKQHTVTTTTKGENFTETDVKM    Saimiri sciureus
ENMYRYPNQVYYRPVDQYNNQNTFVHDCVNITVKQHTVTTTTKGENFTETDIKM    Cervus canadensis M132
                        β2-α2


   210         220         230        240         250
MERVVEQMCITQYRESQAYYQ--RGASVILFSSPPVILLISFLIFLIVG    Odocoileus virginianus wt
MERVVEQMCVTQYKESQAYYDGRRSSSTVLFSSPPVILLISFLIFLIVG    Mus musculus
MERVVEQMCITQYERESQAYYK--RGSSMVLFSSPPVILLISFLIFLIVG    Homo sapiens
MERVVEQMCVTQYQKESQAYYDGRRSS-AVLFSSPPVILLISFLIFLIVG    Mesocricetus auratus
MERVVEQMCITQYEKESQAYYQ--RGSSMVLFSSPPVILLISFLIFLIVG    Macaca fascicularis
MERVVEQMCITQYEKESQAYYQ--RGSSMVLFSSPPVILLISFLIFL      Saimiri sciureus
MERVVEQMCITQYQRESEAYYQ--RGASVILFSSPPVILLISFLIFLIVG    Cervus canadensis M132
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While the primary and secondary structures of PrP^C play a significant role in the species barrier, the specific invading strain is also important. Hamster HY and DY strains, adapted from transmissible mink encephalopathy, share the same amino acid sequence, yet mink are refractory to the HY strain while DY is pathogenic (Bessen et al., 1992). Here we show that Wisc-1 and elk CWD strains can infect Syrian golden hamsters while an emergent CWD strain (H95$^+$) is transmissible to mice, overcoming a well-delineated species barrier and expanding the host range of CWD.

4.3 Materials and methods

4.3.1 CWD inocula

Brain homogenates were derived from clinically-affected white-tailed deer (*Odocoileus virginianus*) of defined genotypes: homozygous Q95 G96 (wt/wt), heterozygous Q95 S96/wt (S96/wt), heterozygous H95 G96/wt (H95/wt), and H95 G96/Q95 S96 (H95/S96) (Johnson et al., 2011; Duque Velásquez et al., 2015) (Figure 2.1). A pool from 4 captive CWD-positive 132M/M elk (*Cervus candensis*) was used to compare properties of the strains CWD2 (Angers et al., 2010), this elk pool was a kind gift from Catherine Graham. Brain homogenates from an uninfected deer served as negative controls.

4.3.2 Animal Bioassays

This study was carried out in accordance with the guidelines of the Canadian Council on Animal Care. The protocols used were approved by the Institutional Animal Care and Use Committees at the University of Alberta.

Weanling C57Bl/6 mice were intracerebrally inoculated with 30ul of 1% brain homogenate. Weanling Syrian Golden hamsters (*Mesocricetus auratus*) were intracerebrally inoculated with 50 µl of 10% brain homogenates (elk CWD was passaged at 1% brain homogenate). Mock-infected controls received brain homogenate from unaffected, PrP-res negative deer. Mice and hamsters were monitored for onset of clinical signs and disease progression. Individual incubation periods are expressed as the number of days post-inoculation (dpi) and were calculated from the time that the mice were inoculated until the time that clinical disease was established. Experiments were terminated at 708 dpi (mice) and 659 dpi (hamsters); all remaining animals were euthanized and assayed for PK-res PrP.

4.3.3 Immunoblot analysis

Brains were collected from mice and hamsters, flash frozen and stored at -80ºC until analysis. Tissues were homogenized to 10% (wt/vol) in sterile water using a tissue disruptor (Omniprep) and disposable homogenization tubes and beads. For the proteinase digestion reactions, 70 µg total protein was treated with 50 µg/ml of proteinase K (Life Technologies) for 30 min at 37°C. Reactions were terminated by boiling the samples in 2.5X Laemmli buffer (150 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 25% glycerol, 5% [wt/vol] SDS, 12.5% ß-mercaptoethanol) at 95°C for 10 min. Samples (10 to 15 µg) were resolved on 12% NuPAGE bis-Tris gels (Life Technologies) and transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore). The membranes were blocked for 1 h at room temperature with 5% (wt/vol) non fat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST). Detection was performed using primary monoclonal antibody 3F4 (0.2 µg/ml diluted 1:10,000 in 5% [wt/vol] nonfat dry milk in TBST; for Hamster PK-res PrP; a kind gift from Richard Rubenstein) or

SAF83 (0.2 µg/ml diluted 1:10,000; for mouse PK-res PrP; Cayman), secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibody, and chemiluminescent substrate (diluted 1:10,000; Life Technologies). Images were acquired on X-ray film (Super Rx; Fujifilm).

4.4 Results

We previously identified two CWD strains in experimentally infected white-tailed deer (Duque Velásquez et al., 2015). These strains, Wisc-1 and H95[+] exhibit distinct biological properties in deer and transgenic cervidized mice. To further ascertain their host range, we transmitted white-tailed deer isolates (wt/wt, 96S/wt, H95/wt and H95/S96) and elk CWD into hamsters and mice. On primary passage, mice inoculated with CWD prions containing H95[+] (H95/S96 deer homogenate) succumbed to prion disease with an average incubation period of 575±47 days, while 5/7 mice inoculated with H95/wt deer homogenate developed clinical disease at 692±9 days (Table 4.1). Mice inoculated with Wisc-1 prions (wt/wt or S96/wt deer homogenates), elk CWD or uninfected deer homogenates were euthanized at 708 days post-infection (dpi) without signs of disease. Clinical signs of H95[+] CWD in C57Bl/6 mice included ataxia, lethargy, tail rigidity, and dermatitis. PK-res PrP was present in all mice infected with H95[+] prions (Figure 4.2) demonstrating that H95[+] is 100% penetrant. PK-res PrP was not, however, detected in mice infected with Wisc-1 prions from wt/wt or S96/wt deer or mice infected with CWD2 from the elk pool. H95[+] infected mice had lower levels of PK-res PrP compared to mouse-adapted scrapie strains (RML, ME7, 22L) (Figure 4.3).

Table 4.1. Intracerebral inoculation of CWD in Mice (C57BL/6). Inocula was derived from clinically affected white-tailed deer expressing different PrP^C primary structures. The strain CWD2 (Angers et al., 2010) was derived from a pool of four elk affected with CWD. This Alberta elk CWD pool was provided by Catherine Graham. For mice (C57BL/6) the inocula consisted of 1% w/v brain homogenates. All hamsters received 10% w/v brain homogenates with the exception of those inoculated with elk CWD (1% w/v). Some transmissions resulted in subclinical infection as demonstrated by the presence of PK-res PrP (+).

CWD	N	C57BL/6		
		Clinical PrP-res +	Subclinical PrP-res +	Incubation Period (dpi)
Deer wt/wt	6	0	0	——
Deer wt/S96	6	0	0	——
Deer H95/wt	7	5	2	669, 671, 706, 706, 706
Deer H95/S96	7	7	0	306, 593, 593, 593, 593, 673, 675
Alberta Elk Pool(CWD2)	4	0	0	——
Uninfected deer	2	0	0	——

Note: Animals euthanized due to intercurrent disease were censored.
Mice with no signs of disease were observed for 659 dpi.
Control mice were inoculated with brain homogenates from prion free wt/wt deer.

Figure 4.2. PK-res PrP in the brain of C57BL/6 mice after primary passage of the H95$^+$ CWD strain. Only animals infected with the H95$^+$ strains contained PK-res material. The number of positive animals is summarized in Table 4.1.
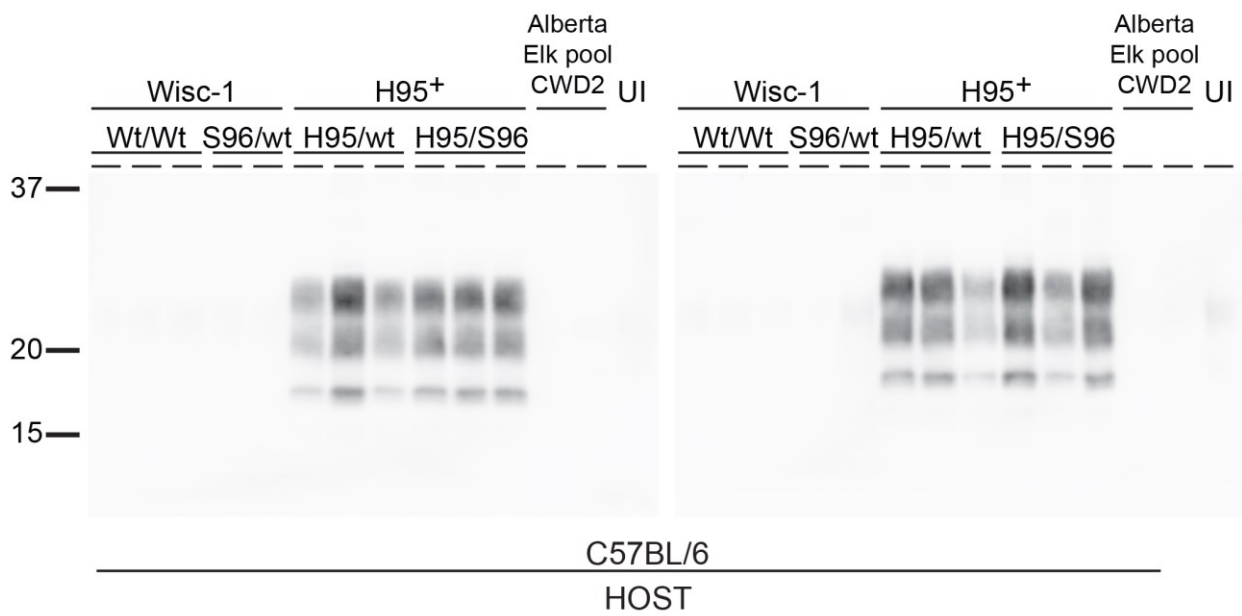
Figure 4.3. Comparison of PK-res PrP from C57BL/6 mice infected with various prion strains. Mice infected with the H95$^+$ CWD strain presented with lower levels and glycotype pattern that mouse-adapted scrapie strains. Brain homogenates were digested with 50 ug/ml of proteinase K. Ten µl of the PK-digested samples was loaded.
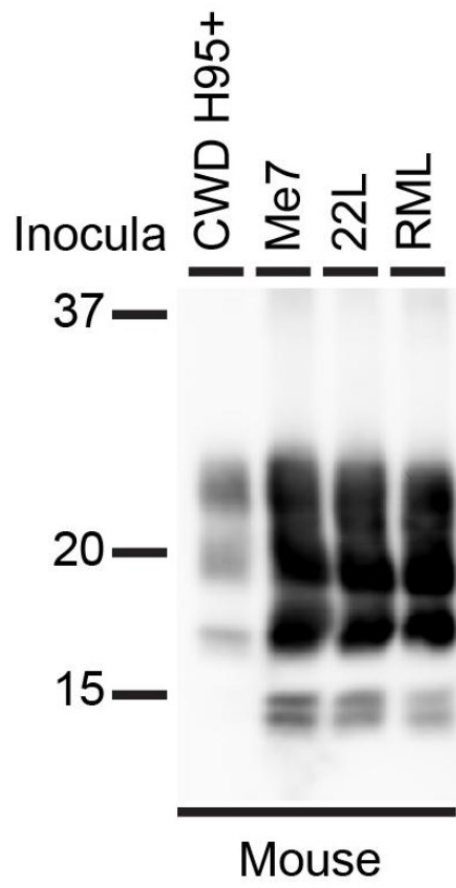
Wisc-1 CWD was preferentially transmitted to hamsters upon primary passage (Table 4.2, Figure 4.4). Wt/wt CWD caused clinical disease or subclinical accumulation of PK-res PrP in exposed hamsters. Transmission of S96/wt and H95/wt CWD prions resulted, primarily, in subclinical disease. Inoculation of elk CWD prions resulted in clinical disease in two out of five hamsters and subclinical disease in one animal. The H95/S96 isolate transmitted inefficiently with only one of eight hamsters having PK-res PrP. This subclinical infection may be due to residual Wisc-1 in the H95/S96 deer isolate. Hamsters inoculated with uninfected deer brain homogenate did not show signs of prion disease nor did they accumulate PrP-res. The PrP-res profiles of hamster adapted CWD was more similar to HY or 263K than DY (Figure 4.5). Clinical signs of CWD in hamsters are initially lethargy and, upon arousal, retrocollis. Dystonic movement included ataxia and tremors. Hyperesthesia was not observed.

Table 4.2. Intracerebral inoculation of CWD in hamsters. All hamsters received 10% w/v brain homogenates with the exception of those inoculated with elk CWD (1% w/v). Subclinical infections were more common in hamsters as demonstrated by the presence of PK-res PrP (+) in various animals euthanized with no clinical signs at the end of the experiment.

| CWD | Hamsters | | | |
	N	Clinical PrP-res +	Subclinical PrP-res +	Incubation Period (dpi)
Deer wt/wt	8	3	5	652, 653, 653
Deer wt/S96	8	1	4	634
Deer H95/wt	8	1	6	652
Deer H95/S96	8	0	1	——
Alberta Elk Pool(CWD2)	8	2	2	673, 719
Uninfected deer	8	0	0	——

Note: Animals euthanized due to intercurrent disease were censored. Hamsters with no signs of disease were observed for 708 dpi. Hamsters infected with elk CWD recived 1% BH and were kept for 729 dpi. Control hamsters were inoculated with brain homogenates from prion free wt/wt deer.

Figure 4.4. PK-res PrP in the brain of hamsters after primary passage CWD various CWD strains. Hamsters inoculated with CWD homogenates derived from deer expressing wt-PrP^C were more likely to test positive for PK-res PrP. In animals receiving the H95/S96 strain, only one subclinical animal accumulated PK-res PrP. Asterisks denote the same sample resolved in both gels for the purpose of comparison.

	Ham CWD P3	UI	CWD wt/wt	CWD S96/wt	CWD H95/wt	CWD H95/S96	Alberta Elk pool CWD2	

50KDa

37KDa

25KDa

20KDa

15KDa

*

*

50KDa

37KDa

25KDa

20KDa

15KDa

MW

MW

Hamster

HOST

Figure 4.5 Comparison of PK-res PrP from hamsters infected with various prion strains. PK-res material from hamsters infected with CWD, hamster-adapted TME and hamster-adapted scrapie. Asterisks denote subclinical samples.
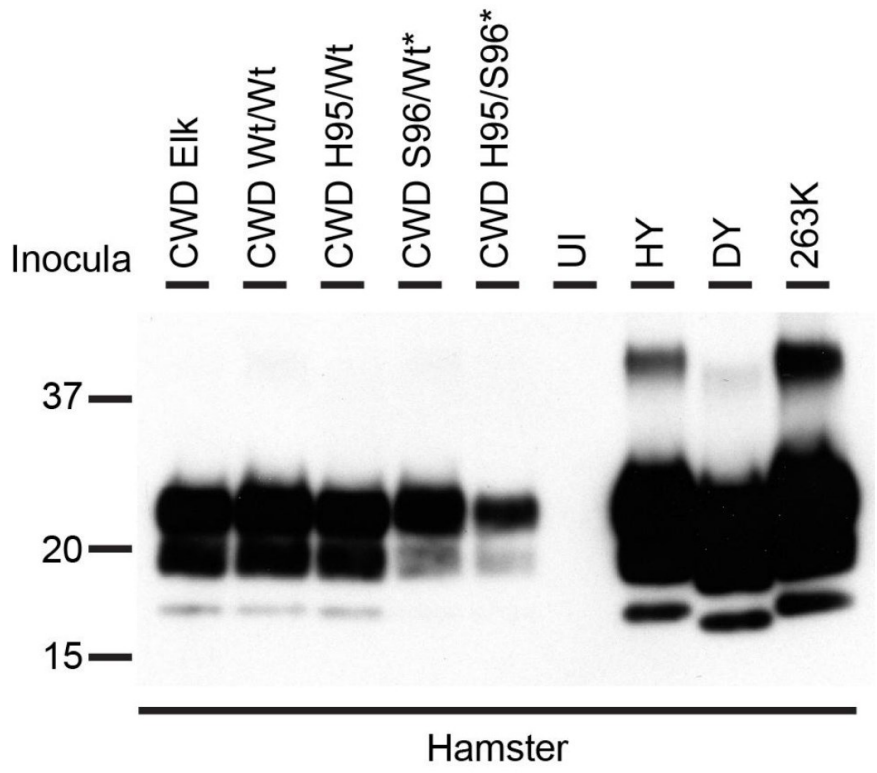
4.5 Discussion

A novel CWD strain was generated upon transmission of Wisc-1 CWD into deer expressing H95-PrP^C (Duque Velásquez et al., 2015). Deer containing H95-PrP accumulate a unique CWD strain (H95$^+$) that exhibits distinct PrP^{CWD} biochemical properties and which efficiently transmits into hosts expressing S96-PrP (Duque Velásquez et al., 2015). Given the ability of H95$^+$ CWD prions to infect S96 transgenic cervidized mice (previously shown to be refractory to CWD prions (Meade-White et al., 2007; Race et al., 2011]), we determined the susceptibility of two commonly used rodent models, hamsters and C57Bl/6 mice, to Wisc-1 and H95$^+$. The susceptibility of C57Bl/6 mice to H95$^+$ CWD and resistance of these mice to the Wisc-1 strain supports the observation that adaptation of Wisc-1 CWD in deer expressing H95-PrP^C generates a new prion strain capable of infecting a new host, mice.

Mule deer, white-tailed deer and elk CWD isolates from Colorado transmit inefficiently into hamsters (Raymond et al., 2007; Bartz et al., 1998; Kurt et al., 2009; Williams and Young, 1992). Successful transmission of Wisc-1 into hamsters indicates this strain is distinct from Colorado CWD strains (Raymond et al., 2007). The transmission of Wisconsin CWD into cattle and fallow deer and inefficient transmission of Colorado CWD provides further evidence of the distinction between Colorado and Wisconsin-derived CWD prions (Hamir et al., 2005; 2007; 2011). The differential transmission of H95$^+$ and Wisc-1 into mice and hamsters is an example of strain tropism to different host species. Together these data demonstrate the existence of multiple strains of CWD (Duque Velásquez et al., 2015; Angers et al., 2010; Perrott et al, 2012; Race et al., 2002b; Lafauci et al., 2006) circulating across North America.

Successful interspecies transmission is, at the molecular level, dependent on the compatibility of the invading prion conformers and structural determinants imposed by host PrP^C. Host species containing PrP^C molecules with a flexible α2-β2 loop (mice, cattle and humans) are hypothesized to be incompatible with PrP^{Sc} molecules derived from species containing a rigid loop (deer and elk) (Gossert et al., 2005; Gorfe et al., 2007; Sigurdson et al., 2009; Sigurdson et al., 2010; Bett et al., 2012; Kurt et al., 2014; Kurt et al., 2015). Our data shows that prions from a prototypic rigid loop species (deer) can transmit to a flexible loop species (mice). The transmission is strain-dependent. H95$^+$ overrides the conformational restriction imposed by the mouse PrP flexible loop that Wisc-1 and elk CWD cannot overcome suggesting that the invading prion strain is a dominant contributor to the species/transmission barrier. How the N-terminal amino acid polymorphism (Q95H) affects the conformation of PrP, altering the deer to mouse transmission barrier, is unknown. Further structural studies may clarify the impact of N-terminal residues on α2-β2 loop rigidity.

The transmission of H95$^+$ CWD prions to mice further confirms the importance of specifying prion strain when defining species barriers. Transmission experiments of CWD into macaques (Race et al., 2009; 2014) and transgenic mice expressing human PrP (Kurt et al., 2015; Wilson et al., 2012) suggest a considerable transmission barrier to CWD prions. Importantly, all of these studies used CWD isolates from the Colorado enzootic foci and none were derived from deer expressing the H95-PrP^C. Successful infection of a flexible loop species (mice) with H95$^+$ CWD raises concerns for the potential pathogenicity of H95$^+$ prions to other flexible loop species, including humans.

The increasing prevalence of CWD is selecting for cervids with resistance alleles (Robinson et al., 2012a; Williams et al., 2014), such as S96 and H95. Importantly, genetic resistance to a given prion strain selects for the emergence of novel prion strains with altered properties, i.e., H95$^+$. The iterative transmission of CWD prions in cervids with protective alleles of PrP^C and the consequential emergence of new CWD strains highlights the dynamics of the CWD panzootic and the importance of characterizing the host range of emergent CWD strains.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Transmission barriers and CWD strains in wild cervids

Variable pathological phenotypes are observed in all mammalian species affected by prion diseases. The underlying cause for these different disease presentations has been associated with multiple prion strains that can reproducibly propagate their specific disease traits (Pattison and Milson, 1961; Bessen and Marsh, 1992a). Unlike viral strains that encode their diversity through nucleic acid sequence variation, prion strains lack this kind of molecule and their basic structure is composed of PrP^{Sc} (PrP^{CWD} in cervid infections), an aberrant form of the host-encoded PrP^{C} (Prusiner, 1982a, 1982b). The current understanding suggests that prion strain diversity is encoded in the conformational variants of the PrP^{Sc} produced during infection (or sporadic disease in the case of humans) (Bessen and Marsh, 1992b; Safar et al., 1998). Also, prion strains sharing the same amino acid sequence produce PrP^{Sc} molecules and assemblies with different properties (Ayers et al., 2011; Safar et al., 2015; Hill et al., 1997). Strains can shift and/or adapt upon passage between hosts expressing different PrP^{C} primary structures resulting, in some occasions, in "mutations" that favour the expansion of the host range (Kimberlin et al., 1987; 1989; Bartz et al., 1998). CWD prions are spread among cervids species expressing different *PRNP* genotypes that code for different PrP^{C} primary structures. The transmission cycle, which involves interactions between cervids, as well as environmental persistence of CWD infectivity, sustains its continuous spread.

The immediate questions that emerge from these properties are: 1) what is the effect of cervid PrP^{C} polymorphism on circulating CWD strains at the PrP^{CWD} level and 2) the consequences of inter-sequence prion propagation on the biological properties of CWD strains?

The effects I described in Chapters 2-4 indicate deer PrP^C polymorphisms have a role in the diversification and host selection of cervid PrP^{CWD} conformers. Most notably, a novel CWD strain ($H95^+$) was identified in deer expressing $H95-PrP^C$ that could infect transgenic mice expressing $S96-PrP^C$. This data suggests that deer carrying the H95 polymorphism accumulate prions that could be more pathogenic to deer with *PRNP* genotypes that are considered resistant. Deer expressing $S96-PrP^C$ have slower progression following exposure to CWD (Johnson et al., 2011; Miller et al., 2012) and this response to disease is thought to provide a reproductive advantage compared to other deer genotypes (Robinson et al., 2012a).

One potential implication of my studies is that as the frequency of the S96 allele rises in response to CWD infection, the selective pressure created by the transmission barrier of $S96-PrP^C$ will favour the adaptation of strains with distinct properties (i.e. $H95^+$). Given that CWD has become enzootic in cervid populations, the continuous transmission could, over time, increase the pool of circulating strains. One immediate consequence of this is that cervids could be naturally infected with more than one CWD strain. Transmission studies in transgenic mice suggest mule deer from Colorado and Wyoming could carry at least two CWD strains (CWD1 and CWD2) (Angers et al., 2010). In the same study, only one strain (CWD1) was detected when a wt/wt white-tailed deer isolate was transmitted. This specific isolate was sampled in Wisconsin, where the CWD epidemic, when compared to Colorado and Wyoming, is a relatively recent event. My transmission studies of CWD prions derived from a wt/wt white-tailed deer resulted in the identification of a strain denoted as Wisc-1 due to its genotype similarity with the parental strain (Duque Vélasquez et al., 2015).

My findings have encouraged new research directions. In one of those, Wisc-1 (wt/wt deer CWD) was transmitted into tg1536, the transgenic mice line used to identify CWD1 and CWD2 (Angers et al., 2010). Inoculation of wt/wt CWD into tg1536 resulted in the propagation of a single strain, supporting our conclusions this isolate is composed of the Wisc-1 strain only (Data from Dr. Sabine Glich at University of Calgary, manuscript in preparation). Unlike transmission of mule deer isolates that resulted in different incubation periods and production of two strain-specific PrP^{CWD} deposition patterns in brain (CWD1 and CWD2; Angers et al., 2010), transmission of Wisc-1 in tg1536 resulted in a single prion disease phenotype.

It has been suggested that amino acid differences between elk and deer can alter the stability of CWD strains following interspecies transmission (Angers et al., 2010). To explore the effect of elk PrP^{C} in the propagation of characterized deer CWD strains, I initiated experiments in transgenic mice expressing elk PrP^{C}. Unlike deer, elk encode glutamic acid (E) instead of glutamine (Q) at amino acid 226. This ongoing study has resulted in the graduate thesis work of a former Masters student at the University of Alberta (Jeff Narayan). Briefly, following first passage of the experimental CWD isolates described in Chapters 2-4, we identified different lesion profiles and patterns of PrP^{CWD} deposition in tg-elk after inoculation with H96/S96, H95/wt or S96/wt CWD. Transmission of wt/wt CWD resulted in a single neuropathology profile, suggesting this isolate is composed of a single strain. An elk CWD isolate served as a control. I have initiated second passages of tg-Elk that presented with variable phenotypes. The neuropathology and PK-res PrP^{CWD} profiles will be compared for the different passages.

5.2. Examining white-tailed deer CWD

The source of the parental strain (Wisc-1) used to the experimentally infect the white-tailed deer described in this thesis (See Materials and methods), was obtained after the first Wisconsin deer tested positive for CWD in 2002. The two deer (animal codes 54344 and 74792) that served as inocula (Wisc-1 isolates) for experimental infection (Johnson et al., 2011) were harvested during November and December of 2002 (Wisconsin DNR; individual deer test results can be confirmed). In 2002, the cervid population of Wisconsin was solely composed of white-tailed deer, excluding the potential effects of PrP^C polymorphism from other cervid species. Given that by 2003 the majority of CWD infected deer (86.5%) were homozygous wt/wt, followed by S96/wt (9.6%) heterozygous (Johnson et al., 2003), it is reasonable to suggest the most common CWD agent was that one selected by the wt-PrP^C. My results suggest that Wisc-1 was the predominant CWD strain circulating in Wisconsin during 2002.

Subsequent analysis of Wisconsin white-tailed deer from the enzootic zone confirmed the wt/wt genotype is the most common in CWD infected deer, supporting the resistance effect of the S96-PrP^C (Johnson et al., 2006a). Epidemiological studies have found a higher frequency of the S96/wt genotype among CWD-negative female white-tailed deer of various age classes, suggesting lower infection and mortally rates compared to the wt/wt genotype (Robinson et al., 2012a). In addition, deer expressing S96-PrP^C have a slower progression following experimental CWD infection (Johnson et al., 2011). The resistance conferred by the S96-PrP^C results in a reproductive advantage that could lead the S96-*PRNP* allele to become more abundant in the population (Robinson et al., 2012a).

Transmission studies of more contemporary samples from Wisconsin have shown that, upon transmission of wt/wt CWD into meadow voles (*Microtus sp.*), only one strain is identified, however, when S96/wt isolates were propagated, at least three additional strains were observed (Dr. Christina Carlson, conference communication Prion 2016). One scenario my studies suggest is that as the Wisconsin epidemic has progressed, the circulating strain pool has become more heterogeneous because of emergence of new strains resulting from transmission barriers imposed by "resistance" PrP^C polymorphisms. Another hypothesis is that as deer expressing S96-PrP^C become more abundant in the population, the H95$^+$ strain emerged.

In Chapter 3 I showed that, similar to deer, tg60 mice are resistant to Wisc-1 CWD infection by the effect of the S96-PrP^C transmission barrier. I have initiated studies to evaluate the current strain composition of Wisconsin CWD isolates (samples were provided by Dr. Carlson). Transmissions into tg33 (wt-PrP^C) and tg60 mice were initiated in December of 2016 with blinded samples. The propagated CWD agents will be characterized accordingly with the methods described in Chapter 2 and 3. If these isolates contain the H95$^+$ strain, tg60 mice should develop prion disease and the characteristic pathological phenotype.

My studies point to the search for other H95 deer isolates and the characterization of CWD agents associated with other deer PrP^C polymorphisms; the former can prove difficult given the frequency of this allele (around 2%; Johnson et al., 2006a). However, Dr. Mike Samuel (University of Wisconsin) has offered access to his archive of CWD and DNA samples of the Wisconsin enzootic zone. The *PRNP* gene of samples obtained at different times during the

epidemic would be sequenced for the presence of *H95-PRNP* and other allelic variants, the corresponding obex would be confirmed and analyzed for PK-res PrP^{CWD} and transmission experiments in tg60 (S96-PrP^{C}) mice would be initiated. These studies would be complemented with other *in vitro* methods that would help narrow down the selection of samples to be transmitted. Some of these methods were described in Chapter 2.

A number of ongoing projects have been initiated due to the findings described in this thesis. Using the differential susceptibility of tg33, tg60 mice and hamsters, I have initiated a characterization of CWD isolates from free ranging mule deer and white-tailed deer of CWD endemic regions of Alberta and Saskatchewan. A first group of CWD samples (N=10) were from clinically affected cervids identified by surveillance efforts. These samples represent various time points since CWD was described in Canada. Although all of the samples were from deer homozygous for *wt-PRNP*, transmission differences were observed upon passage in tg33 mice. In addition, the PK-res PrP^{CWD} profiles of these samples have also presented with variable resistance to proteolysis and electrophoretic patterns. The PrP^{CWD} conformational stability and antibody epitope recognition of PK-res PrP^{CWD} will be analyzed.

I also initiated experiments examining the interspecies transmission of the CWD isolates. These transmission experiments and the characterization of PrP^{CWD} is being performed in collaboration with Elizabeth Triscott (PhD student). Following first passage into hamsters, these studies have identified at least two different PK-res PrP profiles. The characterization of the PrP^{CWD}

biochemical properties and the transmission patterns in transgenic mice and hamsters will provide

a snapshot of the CWD strains circulating in Alberta and Saskatchewan.

To expand on the effect of other cervid prion protein polymorphisms, I am characterizing

experimental elk isolates differing at amino acid 132 (M/L). So far this study has revealed

variable incubation periods following transmission to transgenic mice expressing elk PrP^C. These

differences are unlikely to be associated with infectivity levels as the isolates were derived from

elk in the clinical phase (Dr. Catherine Graham, Canadian Food Inspection Agency). These

isolates were also transmitted into transgenic mice expressing deer PrP^C.

5.3. Zoonotic potential

Considering cervids are traditionally hunted and venison is a food source, the effect of emergent

strains on the zoonotic potential of CWD could represent a hazard for populations that rely on this

food resource. It has been proposed that variation of the amino acid sequence connecting the beta-

sheet 2 (β2) and the alpha helix 2 (α2) of PrP^C (also known as the β2-α2 loop) controls

interspecies transmission (Sigurdson et al., 2010; Kurt et al., 2014). C57BL/6 mice and humans

share this sequence, resulting in production of a flexible loop structure and resistance against

CWD infection (Kurt et al., 2014). Data presented in Chapter 4 confirms the structural barrier

imposed by this flexible loop could be overcomed in a strain dependent manner. Unlike other

CWD agents tested in C57BL/6 mice, $H95^+$ resulted in clinical prion disease. Regarding the

difference between mice and humans at amino acid 168 of PrP^C, a codon implicated in human

resistance to CWD (Kurt et al., 2015), it would be interesting to test if this amino acid change, that

precedes the β2-α2 loop, would abolish the proclivity of H95$^+$ to misfold PrP^C molecules with flexible loop. To test this hypothesis I have initiated transmission studies in transgenic mice expressing the human prion protein. PMCA reactions using brain homogenates from these transgenic mice will be performed.

5.4. Biochemical and structural effects of deer PrP^C polymorphisms

The role of the octarepeat (OR) domain of PrP^C has gained attention for its simultaneous involvement in protective effects or pathogenesis in prion disease (MacDonald and Millhauser, 2014; Lau et al., 2015; Hermann et al., 2015). This domain of PrP^C is known to bind copper and other metals through the histidines in each of the octarepeats and, in doing so, it can adopt various structural arrangements in a concentration-dependent manner (Chattopadhyay et al., 2005). Lower copper occupancy in the OR has been associated with shorter incubation periods, overproduction of C2-PrP and lower levels of total PrP^{Sc} compared with non-transgenic mice (Lau et al., 2015). In Chapter 2 I showed that, H95/S96 deer accumulated lower levels of PrP^{CWD} and produced more C2-PrP. Although this animal survived longer following oral infection (Johnson et al., 2011), the difference in incubation period could be explained by the required adaptation of Wisc-1 in this deer.

Given that codon 95 and 96 are located in the edge of the OR domain, one question that emerges is how these polymorphism affect copper occupancy and conversion of PrP^C. In transgenic mice engineered to develop copper deficiency, prion inoculation has resulted in extension of incubation periods and lower PrP^{Sc} deposition (Siggs et al., 2012). In cell-free conversion experiments, the

presence of copper inhibits *in vitro* fibrillization of recombinant PrP monomers into amyloid

(Bocharova et al., 2005). Also, one ingredient in PMCA conversion buffer is EDTA, a known

chelator of copper (Barria et al., 2012). To further expand on this point, prion plaques in brains of

infected hamsters contain low levels of copper (Johnson et al., 2013). An experiment can be

devised to test the interaction of H95- or S96-PrP^C with copper. Using substrate brain

homogenates from tg60 mice, PMCA reactions would be seeded with Wisc-1 PrP^{CWD} in the

presence of trace amounts of Cu^{2+} (-EDTA conversion buffer) or in its absence (+EDTA, standard

conversion buffer). If the resistance of S96-PrP^C to conversion by Wisc-1 PrP^{CWD} is due to higher

copper occupancy, conversion should occur more readily in the presence of EDTA. To evaluate

CWD isolates more rapidly compared to animal bioassays, I have adapted the amplification of

PrP^{CWD} conformers by PMCA using brain homogenate substrates from tg33, tg-elk and tg60 (with

H95$^+$ as PrP^{CWD} seed) mice. Also a new transgenic line expressing H95-PrP^C was recently

generated at the CPPFD (Drs. Serene Wohlgemuth and David Westaway).

In addition to the increased production of C2-PrP, an additional novel C-terminal fragment, C3-

PrP, was identified following Wisc-1 propagation in the H95/S96 deer. Both C2-PrP and C3-PrP

have the molecular weight of un-glycosylated and mono-glycosylated PK-res PrP^{CWD} in the

H95/S96 deer. Based on epitope-specific recognition and the higher molecular weight, I conclude

that the cleavage that gives rise to C3 occurs towards the N-terminus compared to C2, which

occurs at the end of the OR domain (Figure 5.1).

Transmission studies of BSE into sheep have identified cell-specific N-terminal fragmentation of

prion aggregates that differ from those observed in sheep infected with scrapie (Jeffrey et al.,

2006b). If N-terminal cleavages are induced during interspecies transmissions that result in adaptation, this can be tested. I will inoculate 263K or Sc237 hamster prions into mice; this transmission is not efficient and results in long incubation periods at first passage, however, some animals accumulate PK-res PrP and adaptation can be achieved (Hill et al., 2000; Race et al., 2002a). If N-terminal truncation is increased with interspecies transmission, comparison of PNGase F-treated brain homogenates from interspecies and intraspecies controls would result in differential abundance of C2-PrP or in the generation of other N-terminal cleavage patterns.

Figure 5.1. Graphic representation of N-terminally truncated species in the brain of H95/S96 CWD infected deer, C3 a novel C-terminal PrP fragment. PrP^C and PrP^{Sc} molecules can undergo N-terminal truncations (β- and α-cleavages). Epitope specific antibodies can distinguish the resultant C-terminal products. A) Full length PrP contains the epitopes for all three monoclonal antibodies (Red; 12B2, 8G8, BAR224). B) C3-PrP is recognized by 8G8 and BAR224 but no by 12B2 that does not recognize H95 or S96-PrP (See figure 2.4). B) C2-PrP results from the β-cleavage and is operationally defined by its reactivity with 12B2. Also, in infected mice and humans, C2 is PK resistant (Chen et al., 1995; Yadavalli et al., 2004). C) C1-PrP results from α-cleavages near and within the hydrophobic domain of PrP^C.
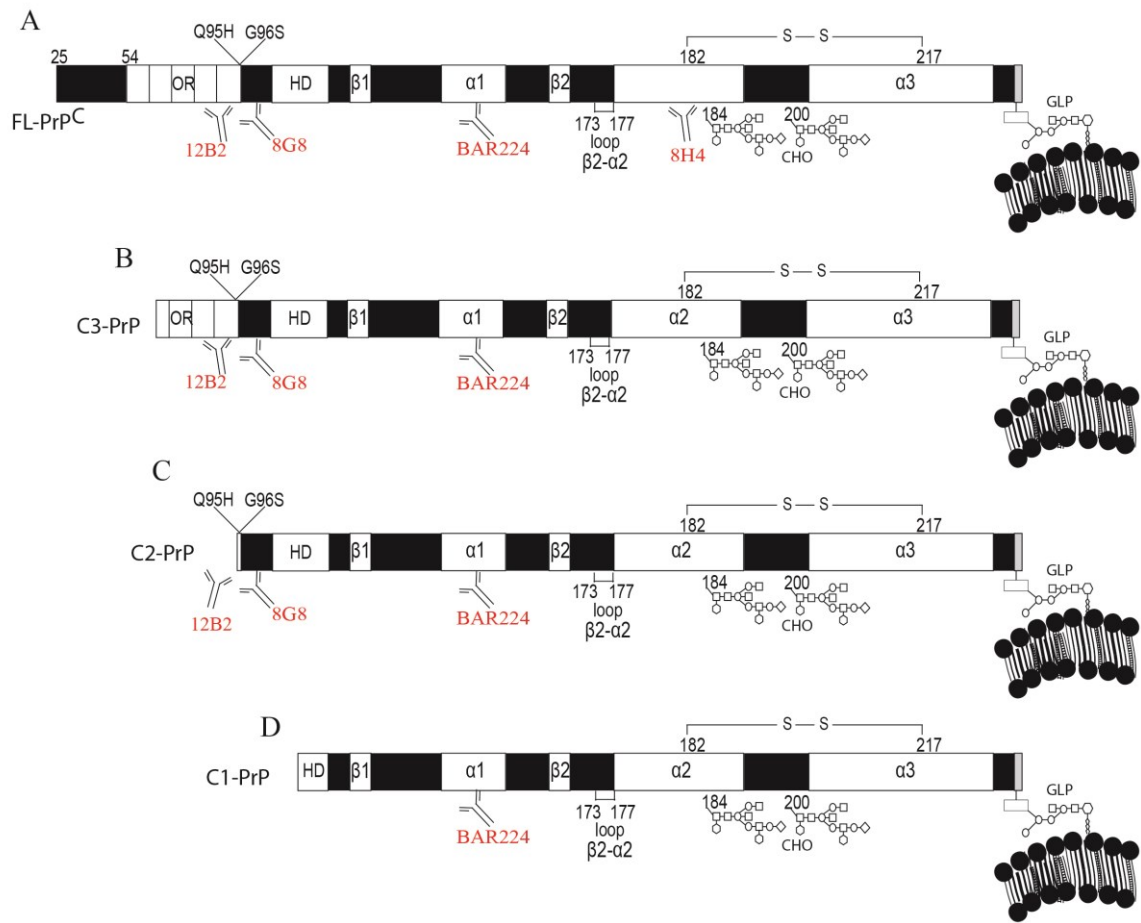
Finally, as presented in Chapter 2, H95/S96, H95/wt and S96/wt deer prions were characterized by variable dissociation and unfolding profiles as compared to wt/wt CWD. These differences suggest co-accumulation of different prion conformers. In collaboration with Dr. Jiri Safar's lab (Case Western Reserve University) we will evaluate this heterogeneity in further detail. Co-existence of prion conformers has been demonstrated in sporadic CJD patients (classified as sCJD1+2) (Haldiman et al., 2013). Using sedimentation velocity fractionation in sucrose gradients, we will attempt to separate these conformational species and evaluate their structural stability in the presence of increasing concentrations of guanidine hydrochloride. If two populations of conformers can be isolated, these results would indicate the co-existence of independent PrP^{CWD} assemblies generated upon transmission of Wisc-1 into heterozygous deer.

5.5 Conclusion

In absence of control measures, we are at the early stages of a larger CWD epizootic in North America, Asia and Europe. CWD will continue to expand its geographic and species range, contributing to the decline of cervid populations. As my research shows, resistance alleles protect only against specific CWD strains and the effects of these PrP^{C} polymorphisms have potential to generate of new PrP^{CWD} conformers, increasing the number of circulating strains. This can lead to the emergence of new biological properties including expanded species tropisms, posing a risk not only to human populations and agricultural species, but also to keystone species.

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