DISSERTATION

PRION STRAIN ADAPTATION: BREAKING AND BUILDING SPECIES BARRIERS

Submitted by

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ABSTRACT

PRION STRAIN ADAPTATION: BREAKING AND BUILDING SPECIES BARRIER

Prions have been an enigma to researchers and agricultural producers alike since their inception. The timing and order of prion disease discovery can be attributed to the scrutiny of the prion protein-only hypothesis. The characterization of bacteria, viruses, and the infectious qualities encoded by their genomes only confounded the hypothetical notion of protein as an infectious agent. Perhaps viral etiology theories could have been disregarded earlier if genetic prion diseases were not quickly overshadowed by experimental transmissibility of the putative infectious protein.

Despite the discordant journey, mounting evidence suggests that prion pathogenesis is caused by the conversion of the normal cellular host protein, (PrP^{C}) into a protease-resistant, abnormal disease-causing isoform devoid of nucleic acid (PrP^{RES}). Importantly, no differences are observed in the primary sequence of PrP^{C} as compared to PrP^{RES} indicating that observable differences between the normal and disease-causing proteins must be conformational. Additionally, even in the absence of nucleic acid, prions are able to infect various hosts differently, suggesting the phenomenon of prion strains. Characteristically long incubation periods and incomplete attack rates, as consequence of primary passage of prion infected material between differing species, but often even within the same species, have been defined as the species and transmission barrier respectively.

Conversion efficiency of infectious prions is most efficient when host and donor PrP^C are identical leading some researchers to believe that heterologous PrP blocks conversion, extending the days to onset of clinical disease. Evidence also suggests that prion protein primary sequence predisposes PrP^C to fold in an un-infectious normal conformation but interaction with a PrP^{RES} conformer, enciphering biological strain characteristics, provides a template for misfolding PrP^C into an infectious conformation. Protein misfolding cyclic amplification (PMCA) has provided additional evidence that PrP^{RES} acts as a template that can convert normal prion protein (PrP^C) into the infectious misfolded PrP^{RES} isoform. PMCA utilizes sonication to break up PK resistant aggregates into smaller prion seeds that may interact and template PrP^C substrate present in the uninfected brain homogenate.

Uniquely, prion disease can be inherited, transmitted, or occur spontaneously. Recently, several investigators have reported spontaneous generation of infectious prions using *in vitro* methods such as PMCA. Additional investigations into host factors needed for efficient conversion and replication has led to the discovery of differences in the propensity of PrP^C misfolding among different species. Several groups have recently suggested that cervid prion protein has a higher propensity for misfolding *in vitro* and *in vivo* as a result of a unique rigid loop identifiable in cervid PrP^C secondary structure. It has been proposed that increased transmission efficiency of cervid prions can be attributed to the presence of this rigid loop.

The principle interest in the current research of this dissertation is to gain deeper knowledge about what fundamental factors play a role in prion strain adaptation, to challenge current theories about prion strain fidelity and to assess species barriers and

prion strain dynamics with the aid of differential mouse models of prion disease. The comprehensive hypothesis of this dissertation is that host factors, including but not solely PrP^C, mediate prion strain adaptation and determine host range and strength of species barriers. We used PMCA, bioassay using transgenic mice expressing variable amounts of PrP^C from mouse and cervid species, and cell culture lines expressing different host PrP^C to address these questions.

We challenged the efficiency and congruency of PMCA by characterizing strain properties of amplified material in parallel with mouse bioassay by: incubation period, PK resistance, glycoform ratios, lesion profiles, and conformational stability. We further wanted to test if PMCA *de novo* generated prions were infectious and what strain properties they would emulate. We hypothesized that the PK resistant material generated with PMCA was infectious and transmissible and possess strain properties reminiscent of other cervid prion strains. Finally, our lab hypothesized that PrP^{RES} conformation enciphers prion strain properties by acting as a template for nascent PrP^{RES} but that host factors also play a role in adapting prion strains derived from a different host and that species barriers can be overcome through this adaptation.

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DEDICATION

I would like to dedicate this work to my parents Tom and Pam Meyerett. They have supported and believed in me no matter what crazy ideas I have approached them with. Their patience, optimism and unconditional love provided me with the confidence and determination to overcome life's obstacles and obtain my goals.

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CHAPTER 1:

INTRODUCTION

History of Protein

The significance of proteins in cellular processes was recognized and reported as early as the 18th century. Protein innovator, Antoine Fourcroy, was the first to recognize albumin, fibrin and gelatin from animal sources and was the first known chemist to postulate that vegetable proteins could be converted into animal protein[1,2]. Nearly a century later, in 1838 Dutch Chemist Gerhardus Johannes Mulder applied the knowledge gained from Fourcroy, and with his own experimentation, biochemically described a protein. [2] Through written correspondence, Mulder took the direction of Swedish colleague, Jons Jakob Berzelius, and named the fundamental substance "protein," after the Greek word "prota," meaning "of primary importance." Mulder further went on to examine Fourcroy's predictions that plant protein could be transferred to an animal unaltered.

Shortly after Mulder published his elemental analysis of these nitrogen-containing animal proteins described fifty years earlier by Fourcroy, Justus Leibig recognized the importance of the protein chemistry field. Liebig was a well-known chemist at the University of Giessen and was fascinated with all things chemistry[2]. It was Liebig's obsession with chemistry that helped bridge the gap between chemistry and related scientific fields such as agriculture, nutrition, physiology, and fermentation[3]. Many advancements and knowledge have been founded on these primary experiments. Researchers now have a richer understanding of protein structure, function, and their

involvement in cellular processes. However, the knowledge gained from these pioneering investigators has not come without controversy. Theories explaining protein structure and function have been widely disputed, especially during primitive experimentation when technological tools were lacking. Perhaps this dissent can be attributed to the fact that so many scientific fields rely on and incorporate a fundamental understanding of protein biology. It is not surprising then, that a protein that defies biochemical and biological paradigms (such as the prion protein) described by our scientific forefathers also has a lengthy history of controversy and ambiguity.

History of Pathogens

Protein science was an important foundation for pathogen discovery in the 18th and 19th centuries, but many aspects of disease, the pathogens that cause them, and the host response to them were not well understood. Sheep scrapie was described in 1732 but little was comprehended about several dimensions of this disease, including etiology, pathology, and mechanism. In 1875 Ferdinard Cohn published an early classification of bacteria [4] while Koch, one year later, announced that anthrax disease is caused by a bacterium[5]. Metchnikoff, prescient in immunology, described cellular immunity in 1882,[6] and 9 years later Paul Ehrlich proposed that antibodies (proteins) are responsible for immunity[7]. In 1892 D.I. Ivanoski was the first scientist to isolate and describe an infectious pathogen, the tobacco mosaic virus (TMV), affecting leaves of plants[8]. In 1898 M.W. Beijerinck championed the idea that the infectious filterable pathogen was of viral etiology[9].

Over the next 30 years experts gained a better understanding of viruses, bacteria, their diseases, and associated proteins. Wendell Stanley made significant

advances in crystallization of TMV that led to high-resolution structures of viral proteins and viral particles that advanced viral science[10-12]. In the 1920s neuropsychiatrists, neurologists, and physicians were introduced to a neurological disorder of unknown etiology that would bewilder them for the next 60 years: Creutzfeldt-Jakob disease (CJD), which was named after its discoverers[13,14]. At this time intraspecies transmission of scrapie was shown to occur by inoculating brain and cerebrospinal fluid from infected sheep [15,16]. Then in the early 1950s, Kuru, another neurological disorder of humans, was being reported in Papua New Guinea, but the etiological agent was baffling scientists, much like CJD and scrapie[17]. Alongside this discovery, Hershey and Chase anticipated that only DNA is needed for viral replication[18] and James Watson, Francis Crick, and Maurice Wilkins biochemically described DNA. [19-23] Only a few years later, Fraenkel-Conrat et al. explained that RNA carried genetic information and had infectivity in TMV, another important discovery in virology[24-28]. In 1958 Crick described the foundation of protein synthesis and defined the "central dogma of biology;" information coded by nucleic acid can be synthesized or transferred but proteins cannot transfer information[29].

The 1950s and 1960s proved important in revealing characteristics of neurodegenerative diseases caused by an undetermined pathogen. Research into animal and human neuropathologies was gaining momentum thanks to the work of Gajdusek, Gibbs, Klatzo, Hadlow, Zigas and Sigurdsson. Pattison reported that Cuille and Chelle hypothesized that scrapie was caused by a 'slow virus' as early as 1938. However, the first published hypothesis of this was in 1954 when Sigurdsson reported the idea of sheep scrapie being caused by a 'slow virus' due to the long incubation, an important insight into what might be causing these encephalopathies in sheep (and

humans)[30]. Collaborative efforts in 1959 revealed that kuru was linked to scrapie and CJD[31,32]. During this time Hadlow proposed that kuru was transmissible like scrapie and he further anticipated (based on Sigurdsson's 1954 study) that kuru was a 'slow virus.' He recommended infecting chimpanzees with kuru[31]. In 1966 Gajdusek, Gibbs and Alpers completed these experiments in chimpanzees and repeated them two years later with CJD, leading to the discovery that both diseases were transmissible after intracerebral inoculation[33,34]. Research of viruses and their pathogenicity continued to intrigue the scientific community, as did the transmissible spongiform encephalopathies. Earlier findings that RNA encoded genetic information and was infectious[25-27] overwhelmingly influenced the first theories stating that CJD, scrapie, and kuru were caused by a 'slow virus' [30,31,35, 28].

'*Slow virus' or Protein: birth of the "Prion"*

An important distinction between viruses and what was causing these encephalopathies was mistakenly disregarded early on. In 1944, veterinarian W.S. Gordon used formalin to inactivate looping-ill virus (a common practice) found in brain and spleen of infected animals and used these treated tissues to vaccinate healthy animals[36]. Formalin inactivated the virus but it did not inactivate the scrapie agent that was unknowingly present, and the vaccinated animals died of scrapie two years later[36].

It wasn't until 1966 that the scrapie pathogen received attention for its stability[37,38]. Many experts from different fields tried to inactivate the scrapie agent by ionizing and UV irradiation, extreme heat, high pressures, and other compounds known to inactivate viruses and bacteria[37,39-41, 42,43]. Characterization of the scrapie agent was more easily accomplished with mouse models[44] but masked the earlier observations that CJD had a

familial or genetic component of transmissibility[45-47], another piece of the puzzle that should have revealed striking differences between the scrapie agent and typical pathogens.

Bioassay in mice and then hamsters was a turning point in characterizing the true infectious agent causing the mysterious encephalopathies. Chandler was the first to demonstrate transmissibility and the curious lengthy incubation times upon intracerebral inoculation of scrapie brain homogenate into wild type mice[44]. Intriguingly, Chandler and Eklund shortened the incubation period upon serial passage, a phenomenon we now know to be adaptation[48,49]. A few years later Zlotnik[50] repeated transmission studies in Syrian Hamsters, and incubation periods were shortened to half those of mouse models[51-53]. Rodent bioassays were instrumental in determining the physiochemical properties of the scrapie agent, as well as investigating intra and inter species barriers. Investigators were able to use end-point titration to characterize incubation periods and titers. Stanley Prusiner used these techniques to begin sedimentation experiments to isolate the infectious agent in cell fractions[54]. In concert with the bioassay experiments, a few prescient scientists, Tikvah Alper, I.H. Pattison, and J.S Griffith speculated that the scrapie agent could be of protein origin. Their theories went against the central dogma of biology for which Crick so elegantly provided the foundation for a few years earlier; nevertheless, this inspired exciting, novel, but disputed research.

In 1966 Tikvah Alper used ionizing radiation to try to inactivate and determine the size of the scrapie agent only to conclude that the agent was not easily inactivated with high amounts of UV radiation and therefore must be replicating without nucleic acid[37]. Pattison added further evidence that the scrapie agent was of protein origin based on

his purification experiments[55]. Boldly, Griffith was the first scientist that not only speculated that the scrapie agent was protein but he also offered three mechanisms that might explain how a protein could be infectious and how this infection could be controlled genetically and occur spontaneously[41]. Griffith alluded to the controversy and chagrin some might think his mechanisms brought to the field in the discussion of his 1967 paper: "…the occurrence of a protein agent would not necessarily be embarrassing although it would be most interesting[41]." Several researchers followed in Griffith's footsteps and accumulated data that continued to suggest the scrapie agent was dependent upon protein[56-58]. It was Stanley Prusiner, however, that took the "protein-only" hypothesis to a rebellious new level. Prusiner and colleagues were able to strengthen their argument through successful protein inactivation experiments of the purified infectious agent from diseased animal preparations[59-65]. In 1982 Prusiner coined the term "prion," proteinaceous (only) infectious particle, to describe the infectious scrapie agent, for which he would later win the Noble Prize.

Despite Prusiner's bold attempts to prove the protein-only hypothesis, decades would pass before it became an accepted hypothesis and understandably so; the positing of an infectious and transmissible protein contradicted core scientific knowledge. Prion and rival scientific fields have spent countless years trying to prove or disprove the prion hypothesis. However, further evidence supporting Prusiner and his "protein-only" predecessors was presented when the normal cellular prion protein, PrP^{C}, was shown to be necessary for infection through the use of $Prnp^{0/0}$ knockout mice [66, 67]. Additionally, Prusiner and colleagues provided fundamental evidence that prions are nonimmunogenic, suggesting that the scrapie agent must be a recognized host

encoded protein[68,69]. Moreover, confirmation of spontaneous prion formation from non-infectious material *in vivo and in vitro* and the ability to replicate the infectious particle *in vitro* with the use of PrP^C as a substrate with the protein misfolding cyclic amplification (PMCA) assay provided concrete protein-only evidence[70-78,79,80]. Most of the scientific community now accepts the hypothesis that prions are infectious proteins devoid of nucleic acid.

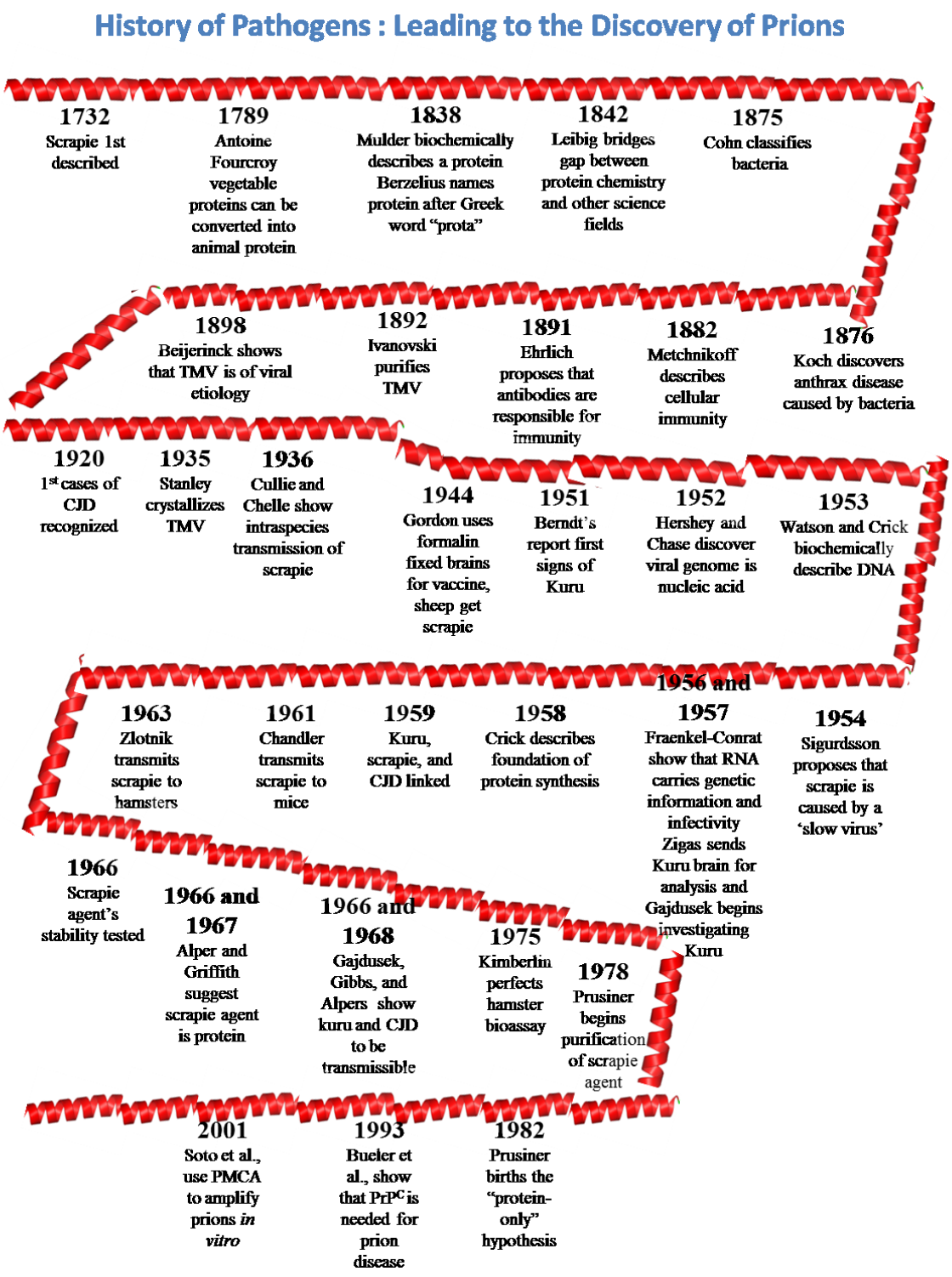

Figure 1.1. Historical timeline of protein discovery and the events in science that led to the protein only prion hypothesis.

Prion Protein Properties

Prions cause a class of diseases classified as transmissible spongiform encephalopathies (TSEs), which characterizes the pathogen well, as prions can be transmitted and vacuoles appear histologically in the brain of infected individuals[81]. Mounting evidence demonstrates that prion pathogenesis is caused by the conversion of the normal cellular host protein, (PrP^{C}) into a protease-resistant, abnormal disease-causing isoform devoid of nucleic acid (PrP^{RES})[58,82]. Importantly, no differences are observed in the primary structure of PrP^{C} as compared to PrP^{RES} indicating that observable differences between the normal and disease-causing proteins must be conformational. Currently, there are no antibodies that can distinguish between PrP^{C} and PrP^{RES} in infected animals without protease digestion. However, investigations of conformation dependent epitopes of the prion protein isoforms are looking promising[83].

Approximately 250 amino acids encode the mammalian prion protein (PrP) which has several distinct domains (Figure 1.2): the amino-terminal signal peptide that is cleaved during posttranslational processing; a highly conserved, hydrophobic octapeptide repeat region; and a hydrophobic carboxy-terminal sequence that signals the addition of a glycosylphosphatidyl-inositol (GPI) anchor[84]. Within the C-terminal globular domain of PrP^{C} there are a few unique properties observed in the structure. During biosynthesis a GPI anchor is attached, associating PrP^{C} with cholesterol-rich lipid rafts at the cell membrane[84,85]. PrP^{C} and PrP^{RES} can be un-, mono-, or di-glycosylated with N-linked oligosaccharide chains that are added at two positions (180 and 196 in mice) initially in the endoplasmic reticulum (ER), but are made more stable within the golgi[86].

Analysis of secondary structure of purified Syrian hamster brain and nuclear magnetic resonance of recombinant Syrian hamster and mouse PrP determined that the C-terminal globular domain is comprised of 3 α-helices (42%) and 2 β-sheets (3%)[87-89]. Conversely, PrP^{RES} is thought to be predominately of β-sheet structure (43%) and therefore, the hallmark of prion disease is the conversion of PrP^{C} α-helical structure to the PrP^{RES} β-sheet isoform. These structural transitions of PrP are responsible for overt physiochemical differences. PrP^{C} is soluble in non-denaturing detergents and fully hydrolyzed in the presence of a serine-protease (proteinase K (PK)) while PrP^{RES} is insoluble and partially resistant to PK, producing an infectious, protease-resistant amino acid core of about 142 amino acids and 27-30 kDa (PrP^{27-30})[59,90]. The second and third α-helices are joined by a disulfide bridge, and α-helix 2 and β-sheet 2 are linked with a large loop, known as the L1 loop, which has unique inter-specific properties (Figure 1.2)[91,92]. The L1 loop has been shown to be exceptionally flexible in most species but is very rigid in deer and elk (Figure 1.2). Variation seen in the L1 loop is thought to be associated with conversion and transmission susceptibility of the host[92]. Unique conservation of PrP structure is observed in the disordered N-terminal domain which contains a glycine-rich octapeptide-repeat region, composed of amino acids PHGGGWGQ, that is thought to bind metal ligands such as copper and manganese[87,93-97, 98].

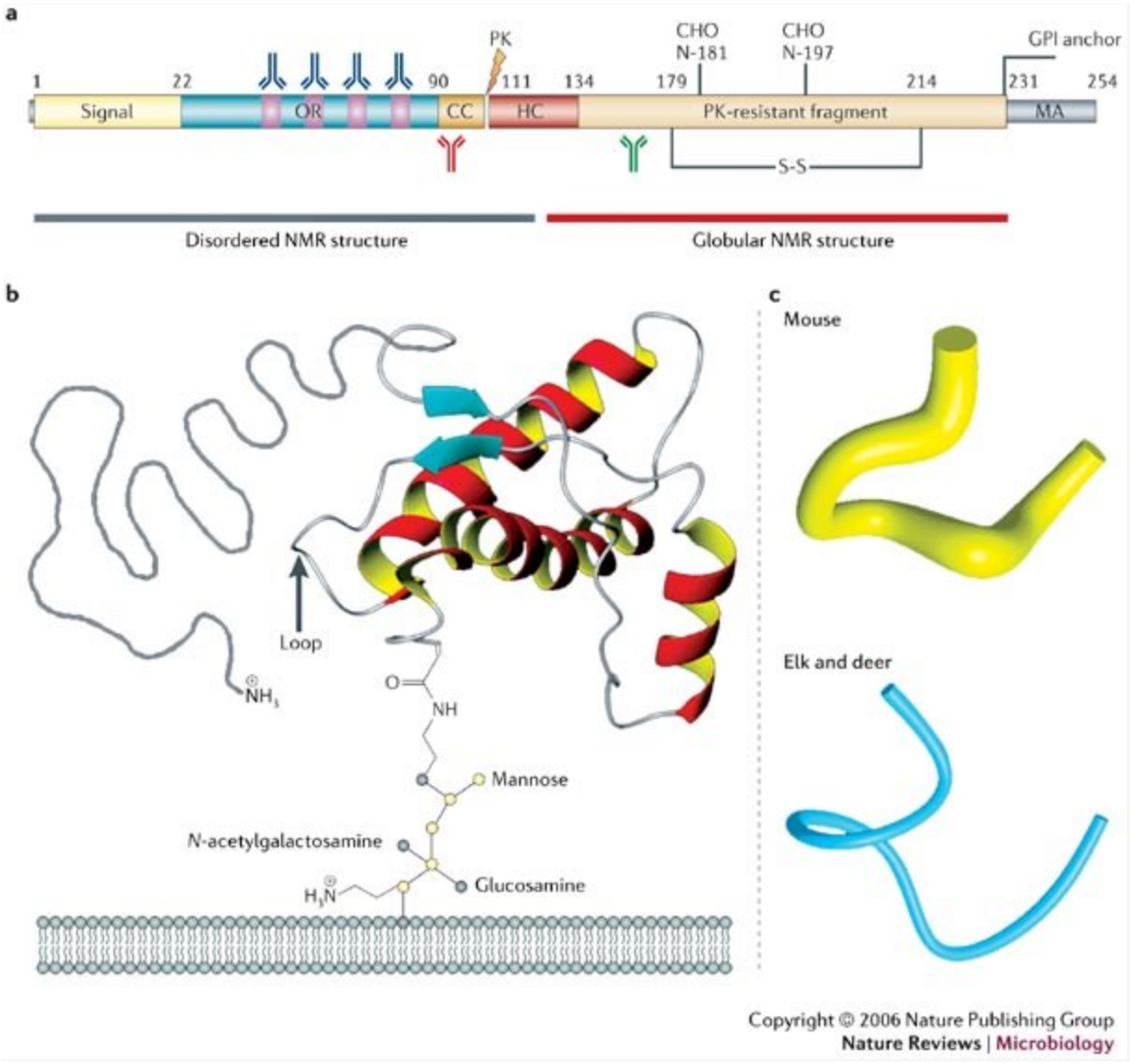

Figure 1.2. (a) Primary structure of PrP^{C} with post-translational modifications. The numbers above indicate the amino acid position respectively. The cleavage site of PK is depicted by the lightening symbol and the consequential resistant core of PrP^{RES} is indicated in gold. **(b)** Representative of PrP^{C} tertiary structure. The loop between the second β-sheet and the third α-helix is indicated by the black arrow. **(c)** Comparison of amino acids 165-172 forming the flexible loop in the mouse and the rigid loop in cervids.

Prion Protein Function

Remarkably, PrP^{C} secondary and tertiary structure is highly conserved in mammals and expression has been identified in many peripheral tissues including skeletal muscle, kidney, heart, and antler velvet[99-101, 102] but is predominate throughout

the central nervous system (CNS) and the lymphoreticular system (LRS). Curiously, knock-out mice that do not express PrP^C are relatively normal so the physiological function is not understood or the function is redundant in other cellular processes. The only role of PrP^C that is absolutely certain is that it is needed for prion disease, as *Prnp* knock-out mice are completely resistant to infection but are completely susceptible upon rescue of the wild type phenotype[103,104, 105].

Many theories have explored the idea that PrP^C must be associated with cell signaling transduction because, as with most cell signaling proteins, it is GPI-anchored in lipid rafts at the cell membrane. Most of the cell signal transduction theories have been based on neuroprotective functions. Chiarini et al., reported that PrP^C conferred neuroprotection to retinal explants by rescuing them from apoptosis[106]. Lopes et al., showed that interaction of PrP^C with stress-inducible protein 1 *in situ* hippocampal neurons could be neuroprotective by activation of cAMP-dependent protein kinase A through the MAPK pathway[107]. Other researchers have suggested that PrP^C offers neuroprotective properties or cryoprotection against harmful agents or oxidative stress by enhancing divalent metal cation (Cu and Zn) dependent superoxide dismutase (SOD)[108, 109, 110]. Wong and Brown et al., convincingly showed that SOD activity correlated with the level of PrP^C expression, and PrP^C contributed to total SOD activity[111,112]. Many experiments have failed to demonstrate that PrP^C is fastidious to any particular function or interaction with proteins. Therefore, the importance and role of PrP^C remains an enigma for prion researchers even though it is ubiquitously expressed in vertebrates[113].

The Significance of Prion Protein (PrP) Genetics

Prion diseases can manifest as genetic, sporadic or infectious conditions, all of which can be associated with the PrP gene on some level. Inherited forms of prion diseases, referred to as Gerstmann–Sträussler–Scheinker syndrome (GSS), familial CJD, and fatal familial insomnia (FFI) disease, account for 10-15% of all prion disease[45,46,114,115]. Mutations in the prion protein gene (*PRNP)* are thought to be responsible for inherited prion disorders and possibly for some sporadic cases[116,117]. Polymorphisms in the gene have been well documented and linked to susceptibility for many prion diseases, most notably CJD and scrapie[118-120].

The PrP gene is a member of the *Prn* gene family. The open reading frame of the PrP gene is located within one single exon and in humans is located on the short arm of chromosome 20 (chromosome 2 in mice)[121]. PrP mRNA is constitutively expressed in neurons, astrocytes, and oligodendrocytes of adult brains, but is more tightly regulated during embryogenesis and fetal development[122-124]. Ford et al. did not see a direct correlation between levels of mRNA in a cell and levels of PrP^C expression, so not all expressed mRNA is being translated[99]. Interestingly, another gene (*prnd)*, which is under the promoter of *Prnp*, has been named “doppel” because it is **do**wnstream and **p**rion **p**rot**e**in **l**ike of *prnp*[125-127]. Doppel maintains a similar structure to PrP but is only expressed in the testis. Interestingly, expression of doppel in the CNS leads to cerebral dysfunction and neurodegeneration[125,126]. The discovery of doppel led researchers to investigate similarities of *Prnp* to other genes, and this resulted in the discovery of *sinc* and *prn-i* genes. Investigators speculated these genes were responsible for short and long incubation differences among mice[126,128-130]. Dickinson published a persuasive paper

suggesting that prion strains encode their own unique disease characteristics, but that host encoded PrP^C, specifically the *sinc and prn-i* genes, regulate the disease progression and pathogenicity[128]. Elegant gene targeting studies by Moore et al. confirmed that *sinc*, *prnp,* and *prn-i* genes were all congruent, and PrP allotypes at codons 108 and 189 were responsible for long or short incubation periods in mice[130].

Certain polymorphisms of *Prnp* have conferred complete resistance to prion disease, most notably in sheep. In 1960, RH Parry adopted the theory that sheep scrapie was a transmissible hereditary disease linked to a recessive gene[131]. Years of sheep breeding records demonstrated that scrapie wasn't hereditary, but susceptibility or resistance to scrapie was. Three polymorphisms have been found in sheep at codons 136 (A/V), 154 (R/H), and 171 (Q/R)[119]. Suffolk sheep heterozygous or homozygous for arginine at codon 171 demonstrated complete resistance to scrapie, while sheep homozygous for glutamine are susceptible[120132-134]. This phenomenon was also demonstrated in Cheviot sheep homozygous for alanine at codon 136 (resistant) or homozygous for valine (susceptible). Curiously, sheep heterozygous at codon 136 were susceptible to disease but the incubation period was lengthened[135]. Goats show redundancy in polymorphic susceptibility to sheep at codons 142, 143, and 240[136,137]. Scrapie is a unique prion disease as it has been eradicated from flocks all over the world by breeding for the resistant polymorphisms. Ironically, however, many of the flocks that are resistant to classical scrapie are susceptibility to other prion strains such as BSE and atypical scrapie, making eradication of all prion diseases in sheep and goats impossible[138,139]. Cervids (deer, elk and moose) also have PrP polymorphisms that may aid in protection against chronic wasting disease (CWD). Telling et al. used

transgenic mouse models to demonstrate that mice homozygous or heterozygous for leucine at codon 132 were resistant to CWD prions while those mice homozygous for methionine were susceptible[140]. Similarly, polymorphisms for methionine to valine in humans at codon 129 renders the host resistant to inherited, sporadic and iatrogenic prion disease[118,141,142]. Transgenic mice have become vital for the progression of prion research and have allowed researchers to investigate transmission efficiency, susceptibility and resistance by manipulating genes.

Exploitative Nature of Transgenes in Prion Research

Animal models have been instrumental in advancing scientific knowledge in all fields. Mouse models in particular, have been an invaluable tool in studying prion biology and disease progression. Without the manipulation of the prion protein gene, much would still be unknown about fundamental prion biology because mouse models abrogated the need for lengthy and costly studies in the natural host.

As described earlier, the use of PrP knock-out mice intensified evidence that prion disease is caused by misfolded protein. Many different strategies have achieved knock-out or knock-in mouse models to aid in the study of normal and diseased prion biology. Bueler used a gene targeting strategy to disrupt the PrP gene in Zurich I mice by using an expression cassette for neomycin phosphotransferase[66]. This resulted in some fused mRNA containing *neo* and residual PrP sequences but no expressed PrP^{C}. Comparably, Jean Manson mutated the gene so that no mRNA or PrP protein could be detected by using a *neo* cassette and a unique KpnI site on exon 3[143]. In addition, Mallucci designed an elegant study that utilized a Cre-*loxP* expression system to create a conditional post-natal PrP null mouse. LoxP sites were under control of the *Prnp*

promoter element, while *Cre*-recombinase was under the control of the neurofilament heavy chain promoter. Therefore, expression of the recombinase during development resulted in the deletion of PrP in neurons[144]. The techniques utilized to establish knock-out or knock-in models of PrP have proven delicate as some gene targets have had deleterious or overexpression effects on different but related genes, resulting in un-characteristic neurological phenotypes[145].

Another concern with using mouse models for prion diseases is that even partially expressed mouse PrP^C can confer protection or resistance to prion disease when inoculated with a strain from an unrelated host, e.g. human, cervid, bovid[146, 147]. It has become a common practice to create transgenic mice on a PrP null background to eliminate any protective effect mouse PrP^C could have on disease phenotype. Host encoded PrP^C plays a pivotal role in transmission efficiency in other species as well. If host PrP^C does not match that of the infectious inoculum, transmission is inefficient, a phenomenon known as a species barrier. For example, wild type mice are resistant to CWD prions but transgenic technologies are able to rectify this problem by knocking out the mouse *Prnp* and inserting a cervid transgene.

Additionally, there is an inverse relationship with the level of the PrP^C expression and incubation periods[148]. Therefore, transgenic mice overexpressing PrP^C from the host of interest succumb to prion disease much faster than those with normal expression levels. Browning et al., constructed a cervidized transgenic mouse (Tg(cerPrP)) that overexpressed (6-8 fold higher) cervid PrP^C and efficiently replicated prion strains from deer and elk. They achieved overexpression by modifying the CerPrP S2 allele nucleotide sequence by site directed mutagenesis[149]. The construction of mice that

overexpress (5-8 fold higher) PrP^C has resulted in an observable shorter disease course and has given researchers the tools to study any prion disease using the same model.

Prion Strain Dynamics

Kuru, CJD, GSS, FFI, scrapie, etc. have distinct phenotypes and strain properties so were not linked to the same infectious agent until 1959. The concept of strains was a novel idea that had not been defined in early prion disease, resulting in a different name for every prion disease despite being caused by the same etiological agent. Historically prion disease nomenclature has added to the complexity of linking prion diseases and discovering unique strains.

Cullié and Chelle were the first investigators to discover scrapie transmissibility by different injection routes into healthy sheep with spinal cord or brain from a diseased sheep. In 1939 they continued their transmission studies demonstrating efficient transmission of sheep scrapie into goats but resulting in different clinical signs[15]. Unknowingly, Chelle expanded the idea of strains in 1942 by describing different clinical signs in goats and sheep naturally infected with sheep scrapie compared to experimentally inoculated goats[150]. During this time, a new transmissible spongiform encephalopathy strain was discovered in farmed mink, transmissible mink encephalopathy (TME). However, TME was not described and characterized until the late 1980s and early 1990s[52,151]. In the 1950s Wilson expanded scrapie transmission studies by serially passaging scrapie material in sheep for 9 passages[152] and subsequently investigated species barriers in mice, guinea pigs, and rabbits. In 1957 Pattison, Gordon and Millson followed suit in goats, demonstrating that goats were even more susceptible hosts than sheep but exhibited a “drowsy” phenotype[150]. They named

the scrapie goat strain SSBP/1. A few short years later, in 1961, Chandler revolutionized prion research by successfully inoculating mice with goat scrapie[44]. Other researchers began to mimic bioassay experiments in mice but were displeased with the lengthy incubation periods paralleling the disease course of the natural host[153]. Zlotnik and Chandler began testing species barriers in different rodents in search of new, more efficient models of scrapie. In 1963 Zlotnik experimented with hamster bioassays, and Chandler challenged rats; both were pleasantly surprised by their success[50,154].

At this time Zlotnik and Rennie developed a well-known scrapie strain, ME7. This strain was derived from a pool of infected Suffolk sheep spleens. Zlotnik and Rennie inoculated mice intragastrically (i.g.) with the spleen pool and created a brain pool from the infected mice which they subsequently passaged intracranially (i.c.) into RIII (C57 Blk6 mouse lineage) mice[155].

A new prion disease of cervids, chronic wasting disease (CWD), was first described in 1967 in captive mule deer[156]. Ten years later, Dickinson characterized 3 strains (22A, 22C, 22L) from mice inoculated with SSBP/1 goat scrapie using incubation periods and vacuole distribution[157, 158]. In the late 1970s and into the late 1980s, RH Kimberlin and colleagues like Walker, Dickinson, and Fraser, investigated strain characteristics more fully using rodent bioassay models and were able to define prion species barriers. Meanwhile, CWD was gaining more attention as Williams described the first case of a TSE affecting a wild population[159,160]. DeArmond and Bruce et al., elaborated on strain characterization in the late 1980s by describing patterns of PrP^{RES} accumulation in the CNS along with lesion profiling[161,162]. In 1986 three dairy cattle in the UK were recognized as having neurologic disease that was later accepted as a new

TSE and named bovine spongiform encephalopathy (BSE)[163]. In 1992 Bessen and Marsh described the isolation of two distinct strains from TME that they appropriately named “hyper” (HY) and “drowsy” (DY) based on the hamsters’ demeanor[164]. HY and DY TME had observable differences not only in clinical signs but also in incubation times and lesion profiles. A few years later, Prusiner et al., established that 5 of 20 mutations known in huPrP are linked to the dominantly inherited human prion diseases fCJD, GSS, and FFI. Additionally, Collinge et al. observed differences in glycosylation patterns between strains adding criteria for characterizing a new strain[165]

A pivotal moment in prion history and identification of prions strains was when the zoonotic potential of BSE was discovered in 1996. An association between people who ate infected beef and a new rapid human TSE was named as variant CJD (vCJD)[166]. One year later, Prusiner’s lab generated novel strains by infecting transgenic mice with different PrP amino acid sequences[167].

In 1998 and 1999 biochemical characterization of prion strains was became an effective approach for describing novel strains as well characterizing the similarities of strains in different diseases (e.g. BSE and vCJD). Additional tools to characterize strains were also being described at this time; Prusiner, along with Groschup, correlated differences in PK stability with conformational changes between strains and postulated that the more sensitive a prion strain is to PK digestion the quicker an animal succumbs to disease[168,169]. At the start of the millennium Jason Bartz and colleagues investigated mechanisms of strain adaptation and selection of TME using the HY and DY strains in a competition hamster model. Remarkably, DY was the selected strain upon first passage but HY was selected in subsequent passages, presumably because HY is a more

efficient replicator[170]. In 2002 more research groups concluded that strain propagation was based on the conformation of PrP^{RES} independent of PrP^{C} sequence[171,172]. It is important to note, however, that many groups conducting strain studies failed to serially passage the new PrP^{RES} strain, overlooking strain adaptation and host factor effects on the nascent PrP^{RES} conformer over time.

In 2006, Aguzzi's laboratory claimed that they were able to cross a strict species barrier and infect transgenic mice expressing high levels of mouse PrP^{C} with a CWD strain[173]. These investigators reported that the original CWD strain characteristics were unchanged after serial passage into the mice, suggesting that strain-specific CWD properties are not altered by host factors. It is important to note that their results could have been a result of spontaneous disease (a common phenomenon in aged, overexpressing mice) as mice did not show clinical signs before 500 days post inoculation (DPI).

In 2008, our group contradicted the Aguzzi's lab findings and demonstrated that prion strains could be adapted (altered) through serial passage using transgenic cervidized mice and a cervid prion strain but failed at crossing the species barrier into wild-type mice. In addition, we were able to show that the cervid prion strain could be more efficiently adapted *in vitro* using protein misfolding cyclic amplification (PMCA). Evidence of adaptation was seen through distinguishable incubation periods, conformational stability, PK stability, glycoform ratios, and neuropathology of mouse-adapted RML[74]. Kimberlin and colleagues paved a persuasive foundation of species barriers and prion strains through their tedious transmission studies. Most of their research resulted in the abrogation or construction of species barriers and novel strains

using species with differing PrP^C amino acid sequences[51-53]. Namely, he used a goat scrapie strain and serially propagated this through mice, rats, and hamsters. He observed incomplete attack rates upon the first passage into hamsters with 300 day post inoculation (dpi) incubation period. However, second passage accelerated disease and all the hamsters were sick in 130 dpi[51]. More recently, Tamguney et al. manipulated elk transgenic mice to chimerically express c-terminal mouse PrP^C and found that species barriers were created and destroyed based on certain elk-to-mouse or mouse-to-elk mutations in C-terminal amino acids[174].

Animal Strains

Scrapie

Scrapie was the first described prion disease, dating as early as 1732 in England. At that time, the disease was only documented by clinical signs, as prions were not even postulated until the mid-1900s. Scrapie is one of the most well studied prion diseases, perhaps because rodent models have been relatively susceptible to scrapie. Scrapie is known to naturally affect sheep, goats, and mouflon. “Scrapie” became the accepted disease name due to the clinical sign of pruritus, evidenced by sheep scraping against objects intensely enough to scrape their wool off. Goats have been observed to preferentially use their hind legs or horns to scratch. In addition to severe itching, animals are ataxic, often trotting with an uncharacteristically high stepping, unbalanced gait. Many people often referred to scrapie as the “trotting disease,” or the “trembling disease” because of observed clinical signs. Weight loss is also observed even though the animals are polyphagic. Infected animals have also been recorded as being restless, anxious, easily stimulated, grinding teeth, excessive

thirst and consequently, excessive micturition[15]. Remarkably, reproductive function is not impaired during disease and many ewes give birth to big, healthy lambs without assistance. However, depending on the animal's disease course, the mother may not have the ability to nurture and care for the lamb once born. Once clinical signs are apparent, death follows in 2 weeks to 6 months[15]. A distinguishing characteristic of prion disease is the lengthy incubation period; signs of scrapie disease are typically seen 18 months to 2 years after exposure.

Scrapie is unique in that it can be eradicated through selective breeding of resistant polymorphic genotypes[135](USDA/APHIS). Suffolk sheep heterozygous or homozygous for arginine at codon 171 have been shown to be resistant to scrapie infection and BSE while maintaining traits producers desire[120,175,176, 177, 178]. Unfortunately, in 1998 an atypical scrapie case (Nor98) was reported in Norway. This scrapie strain has many different pathological features than classical scrapie, but most notable is that sheep considered resistant (ARR or ARQ) to classical scrapie are susceptible to Nor98[177,179-182]. There are reports that Nor98 (atypical scrapie) is restricted to the CNS[183-186]but more recently, there is evidence that there is peripheral expression but not easily detected due to infectious PK sensitive characteristics of this strain[187].

It is still unclear how scrapie started, although most investigators believe that a sheep must have spontaneously developed the transmissible disease. In 1914 McGowan was convinced that scrapie was caused by a parasite because scrapie infected sheep exhibited sarcosporidia in the musculature, but no other evidence links this parasite to scrapie[15]. Transmission of all prion diseases is also unclear but most evidence suggests that scrapie is transmitted orally and vertically from mother to

offspring[188-194, 176, 195-198]. Because infectious prion proteins are so resistant to degradation, it is appropriate to evaluate lateral/horizontal transmission of scrapie. Increasing evidence points to horizontal transmission; introducing uninfected flocks to areas infected flocks once inhabited results in disease a few years later[192,199,200, 201].

Currently there are several ante mortem tests that can be done on lymphoid tissue to diagnose scrapie in sheep and goats: the third eyelid test, rectal biopsy, and tonsil biopsy; however, these are not as sensitive and reliable as testing the brain sections once the animal is dead. In all tests tissues are considered positive if there is immunohistochemical (IHC) staining for PrP^{RES}. Like all prion diseases, positivity is only definitively determined by the analysis of the brain for presence of the characteristic spongiform plaques as well as aggregated prion plaques or fibrils. Scrapie prions are characteristically present in the medulla, particularly in the dorsal motor nucleus of the vagus nerve, which innervates the gut and consequently, prions can be found in the gut throughout Peyer patches in the small intestine[202, 153]. Rodent models share the same neuropathology as the natural host, but IHC staining can be more diffuse with less obvious aggregated punctate plaques[74,203,204].

BSE

Bovine spongiform Encephalopathy (BSE) is the prion disease that affects cattle. BSE has become better known to the public as “mad cow disease” by the media attention it received in the late 1990s when BSE has a zoonotic outbreak into humans. BSE was first diagnosed in the United Kingdom in 1986 in three dairy cattle presenting neurological signs similar to sheep and goats with scrapie. Salient behavioral signs of BSE include aggression, apprehension, and exaggerated responses to stimuli. In

addition cattle are ataxic, have decreased milk production, become very thin, and at end stage clinical disease, become recumbent[205]. Cattle can display clinical signs for 1-6 months before succumbing to the disease. Determining the incubation time for BSE has been challenging due to several factors: transmission events appear to be relatively low between a herd, and most cattle are slaughtered by the age of 6 years old; making a longitudinal study nearly impossible. Anderson et al., correlated epidemiological data, accounting for complications noted above, and statistically predicted the mean incubation period to be around 5 years[206].

Genetic polymorphisms have not been associated with BSE susceptibility as in other species. Some cattle have the presence of an additional octapeptide repeat but this has not been linked to BSE occurrence/susceptibility in tested cattle[194,207-209]. Similar to scrapie, however, atypical BSE strains were identified in 2004. Researchers characterized atypical BSE strains by distinct molecular and neuropathological differences. France and Italy were the first to describe two new strains in cattle that were atypical of BSE well characterized in the UK. Biacabe et al., reported that PrP^{RES} in cattle 8-15 years old had a high affinity to the monoclonal antibody P4, a noticeable change in molecular characteristics from classic BSE as only scrapie prions were recognized by this antibody previously[210]. The French investigators denoted this new strain as H-type because of an observable high molecular mass band of the un-glycosylated PrP^{RES} after PK digestion. That same year, in Italy, Casalone et al., described a low molecular weight un-glycosylated band that they denoted L-type[211]. This strain also differed from classic BSE because it produced a higher ratio of mono-

glycosylated PrP^{RES} rather than di-glycosylated[211]. Atypical BSE cases have since been recognized in Japan,[212]Belgium,[213] and Germany[214].

The origin of BSE has been very controversial but persuasive evidence suggests that BSE arose from cattle eating scrapie-tainted meat and bone meal (MBM) and BSE cases declined dramatically after banning this feed practice[215,216,217,218]. The evidence linking MBM to cases of BSE was so convincing that the practice of feeding MBM was banned in the UK in 1988 and later, in 1996, the practice was banned for all livestock and mammalian-derived meat (not just sheep and cattle) in Europe and in the United States[206,219]. Some researchers believe that BSE began with spontaneously diseased cattle that was rendered into MBM and fed back to cattle and/or that milk replacers that contain infected tallow were fed to calves[220-222]. In 1996, it became evident that BSE was unique from other animal prion diseases; BSE turned into a zoonotic epidemic among people that had eaten infectious beef in the UK (described in the human prion disease section below). BSE has experimentally infected cattle, sheep, pigs, mice, and mink[223-225], [226] and has been linked to natural infections in humans, domestic cats, and captive wild exotic (i.e. zoo animals) bovidae and felidae[227-229, 165,166,230].

There are not currently any ante-mortem tests for BSE because infectious material is not expressed in peripheral tissues. BSE appears to be restricted to the CNS and therefore diagnosis can only be made by identifying the presence of PrP^{RES} in CNS tissues, namely the medulla oblongata. Pathological changes seen in diseased cattle are the characteristic spongiform change, neuronal vacuolation and degeneration, accompanied by astrocytic activation[231]. BSE does not generate robust amyloid plaques but rather marked spongiform change. Because of minimal peripheral shedding of

infectious prions through, meat, milk, blood, etc., horizontal transmission appears to be an inefficient route of transmission[216,232-234].

Chronic Wasting Disease

Chronic wasting disease (CWD) affects captive and free-ranging deer, elk, and moose (collectively known as cervids) in North America at nearly a 30% prevalence rate[235,236]. CWD was first identified as a clinical disease in a captive Colorado mule deer in 1967 by the late Beth Williams but was not recognized as a TSE until 11 years later[156]. In 1980 the disease was recognized for the first time in wild and captive elk[160]. CWD is the only prion disease known to affect a wild population. CWD was named based on observed principle clinical signs such as poor body condition leading to emaciation over an extended disease course of 2 weeks to 8 months[156]. Animals are observed as having behavioral changes, depression, listlessness, polydipsia, polyuria, grinding of teeth, excess salivation, drooping of the head, and (less noticeable) changes in gait/movement[156]. The incubation period of CWD in a natural population is not known, but the National Parks Service, in collaboration with Colleagues of Colorado State University, is currently conducting a longitudinal study with elk to try to further characterize and understand prion progression in the wild (unpublished data). In captivity, animals succumb to disease between 16 months and 4 years after exposure[235]. Evidence suggests that genetic polymorphisms may influence incubation and susceptibility in cervids analogous to other species affected with prion disease.

Despite homology observed in cervid *Prnp, c*ervids contain several species-specific polymorphisms. Mule deer contain a serine/phenylalanine polymorphism at codon 225. CWD infected individuals show a higher prevalence of serine homozygosity

at this codon while those heterozygous or phenylalanine homozygous, though underrepresented in the population, have been reported to have a lower risk of infection or exhibit delayed disease[237]. White tail deer also have a polymorphism containing serine, but deer with serine homozygosity at codon 96 have a decreased risk and/or a delay in disease as compared to serine/glycine heterozygote or glycine homozygote deer[238,239]. Polymorphisms at codon 132 in elk correspond to human polymorphisms at codon 129, but elk are polymorphic for methionine to leucine. Surveyed elk demonstrated a very low frequency of leucine homozygous individuals in a given population. This is unfortunate because these elk, though they are not completely resistant to CWD infection, live longer[240,241]. It is emphatically important however, to consider that those individuals that have a delayed disease onset or do not succumb to disease at all could be asymptomatic carriers of the disease and could be aiding in transmission rather than aiding in eradication of the CWD.

Parallel to other prion diseases, the origin of CWD is ambiguous, though many theories offer explanations. One theory is that CWD originated with a rare spontaneous case that, over time, was able to gain momentum and become efficiently transmitted into susceptible herd-mates. It is also not known if CWD originated in captive animals or in the wild. Epidemiological evidence however, suggests that farmed or captive cervids have been instrumental to the maintenance and spread of CWD. Williams et al. hypothesized that cervids grazing on the same lands as scrapie infected sheep could easily explain interspecies transmission of a TSE[242,243]. Hamir et al., tested this hypothesis by intracranially inoculating scrapie infected brain homogenate into elk, but the results were inconclusive as only half the animals were infected[244]. In a reverse

experiment (CWD infected brain into sheep and cattle), results indicated that animals were poorly susceptible to CWD from mule deer but cattle were highly susceptible to CWD from white tail deer[240,245,246], [247]. Unfortunately, much about the origin and transmission of CWD in a free-ranging wild population remains difficult to elucidate because of the many variables that have to be accounted for in the wild. Through observations of captive cervids, it has become apparent that CWD is transmitted horizontally through an oral route via blood, saliva, feces, urine and environment[248, 249-253, 235,254, 255,256]. CWD poses a new challenge in wildlife management and eradication of such an efficiently transmitted and persistent disease of cervids seems impossible.

Definitive diagnosis of CWD infected animals is accomplished on deceased animals with immunohistochemistry (IHC) of brain tissue, usually the medulla. Several ante-mortem tests have been developed for early diagnosis of prion diseases. All of these tests require biopsy of lymphoid tissue (e.g. third eyelid, tonsil, or rectal tissue) and IHC to identify accumulation of $PrP^{RES_{257-259}}$. Comparatively, these ante-mortem tests are not sensitive, resulting in false negatives of early or unique CWD infections. However, ante-mortem tests can be a powerful diagnostic tool when used in conjunction with more sensitive assays such as PMCA or IHC of brain tissue[260] (Wyckoff, in review).

The neuropathologic spongiosis is evident in CWD infected animals as it is in all prion diseases but is most consistently noted in the medulla oblongata. This brain area has become a standard region for diagnosis and surveillance in captive and free ranging cervids. Distribution of PrP plaques is variable but generally is not detected in the cerebellum. Differentiation of CWD from other prion diseases can be made by

looking at glycoform ratios after PK digestion as CWD has the highest signal in the diglycosylated band[261].

Through increased surveillance and natural transmission, CWD has now been identified in 22 states in the US, two Canadian provinces, and South Korea[262,263]. Consequently, there is a need for a better understanding of CWD transmission routes, efficiency and the environment's role, replication and pathologic mechanisms, and descriptions of strain and species barriers. Because of the unique characteristics and availability of CWD samples, it may be the most appropriate prion disease to study to elucidate the mechanisms prions utilize that continue to elude scientists.

Human Prion Diseases

Kuru

In the early 1950s, an anthropologist couple, Ronald and Catherine Berndt, took interest in the Foré people of Papua New Guinea. During their encounters they learned that Foré tribes practiced sorcery: an act of taking an intimate object (feces, discarded food, hair, etc.) from the intended victim and placing the wrapped object at the edge of a swamp to watch the coincidental decay of the object and the victim[264]. When tribal members came down with an illness it was presumed that someone had used sorcery against them to make them ill. Specifically, the Foré believed those exhibiting the unique signs of kuru were victims of sorcery. Kuru first drew the attention of Vin Zigas in 1955 when he diagnosed a woman with signs of kuru as having 'acute hysteria' and otherwise healthy[264]. A prominent misconception arose that the Foré were succumbing to sorcery in a psychosomatic manner. Individuals with kuru presented with an intense trembling or shaking of the entire body, which is what "kuru" means and why it has been

established as the disease name. Interestingly, investigators, such as Zigas and the Berndts determined that kuru mostly infected woman and children[264]. In 1957 Carleton Gajdusek joined Zigas to study the kuru phenomenon[17]. Kuru was characterized as a TSE in 1968 by Gajdusek and Gibbs et al after he experimentally transmitted kuru to chimpanzees[34, 34,265].

Kuru begins with symptoms of, "lethargy, headache, vertigo and vomiting[264]." The clinical signs include autonomic tremors, unsteadiness and mild ataxia, eventually leading to paresis and severe ataxia, erratic eye movements, and gradually urinary and fecal incontinence and disphagia[17,32]. Sufferers also go through stages of euphoria and uncontrollable laughing alternating with depression[17]. Clinical signs can persist up to 9 months and incubation period of disease is as early as 12 months are as long as 50 years[17,33,266, 267,268].

Increasing evidence suggests that Foré people homozygous for methionine at residue 129 are more susceptible to kuru transmission and experience shortened incubation periods[269, 267,268]. In fact, those individuals that presented with disease long after exposure proved to be heterozygous (M/V) at residue 129. There is also convincing evidence that kuru has caused genotypic selective pressures in Foré tribes resulting in more resistant genotypes[267].

In 1984 Klitzman et al., determined through epidemiologic analysis that the kuru epidemic was result of ritualistic endocannibalism that began in the 1940s and 1950s[266]. Alpers substantiated this evidence with his epidemiologic review in 1992[270]. Endocannibalism is the practice of eating flesh from deceased community members. Kuru only affected the Foré linguistic tribes and close neighbors with whom they

intermarried, and predominately affected the woman and young children in these communities[271]. During cannibalistic rituals, men had the preference of meat to consume and ultimately, women and young children were left to feast on brain[266]. Once endocannibalism was discouraged by the government (and tribes complied) in the late 1950s and early 1960s, kuru incidence decreased (especially in young children)[266,271]. The origin of kuru is unknown. The most plausible hypothesis is that it was introduced spontaneously and cannibalism transmitted the disease efficiently, similarly to the BSE epidemic in cattle.

Kuru neuropathology consists of the characteristic spongiosis change and amyloid plaques with the most severe pathological deficits present in the cerebellum[272] Grossly, atrophy of the cerebellum, specifically of the vermis and flocculonodular lobes, is evident[273]. In 1959, Hadlow was the first to point out the similarities between scrapie and kuru[31], which led to the transmission studies completed in chimpanzees, which continued upon the classification of kuru as a TSE, and ultimately as a prion disease, and the first naturally transmitted human prion disease.

Inherited Prion Diseases

In 1921 Jakob identified Cruetzfeldt Jakob Disease (CJD) in conjunction with a referenced case of Cruetzfeldt's in 1920[13,14]. Ultimately these two investigators contributed to the naming of the invariably fatal neuropathologic disease. CJD can present in a sporadic, inherited, or transmitted form. Sporadic cases of CJD are the most prevalent form of human prion disease but familial CJD was first characterized. Reports about an unknown neurologic disease, now known as Gerstmann-Sträussler-Scheinker, were as early as 1912[274] but it was Gerstmann, Scheinker and Sträussler

who characterized the disease (and named it) in the late 1920s and 1930s[275]. Fatal Familial Insomnia disease wasn't described until 1986 by Lugaresi and Gambetti[276].

The first familial case of CJD (fCJD) was recorded by Kirschbaum in 1924 and more convincingly described by Meggendorfer in 1930[45,277]. The "Backer" family had a long lineage of CJD and aided in the discovery of fCJD phenotypes. There are three different inherited prion diseases currently characterized: Gerstmann-Sträussler-Scheinker (GSS), Fatal Familial Insomnia (FFI), and CJD. These diseases are classified by the phenotype presented in addition to the pathogenic mutation(s) of PrP and their genotype at residue 129 (haplotype). To date, 55 mutations coupled with 16 polymorphisms in the *PRNP* gene have been associated to human prion disease[278]. Clinical signs of inherited diseases can present differently depending on the mutation and genotype but most signs are redundant. GSS, FFI, and CJD all present with cognitive impairment, psychiatric changes (emotional changes), and dementia[279280]. CJD additionally can present with seizures and visual disturbances[281,282]. FFI, as noted by the name, presents with altered sleep-wake cycles resulting in insomnia and these patients often hallucinate and are confused[280]. GSS can present with Parkinsonian-like signs and ataxia.

The incubation period and duration of clinical signs of these inherited diseases rely on the individual's haplotype. Generally GSS disease affects individuals between 40-60 years of age and clinical symptoms are present for 3-6 years on average[283,284]. Clinical signs of fCJD generally appear in 30-55 years and persist for months to years[281,285-288]. FFI presents in patients 40-50 years of age and can persist from 12-16 months[289].

GSS, FFI, and CJD have distinct histopathological differences. fCJD resembles other prion diseases with the characteristic spongiosis and astrogliosis throughout the cortex and deep nuclei[287,290]. GSS is well characterized by intense amyloid plaques present when staining for PrP, while FFI has distinct neuronal loss and astrogliosis in the thalamus and inferior olive but no spongiosis is detected[289,291, 290].

Inherited prion diseases are autosomal dominant and therefore, are easily retained and passed through populations. Researchers have done an incredible amount of work to map out 30 known genetic mutations that can elicit these diseases[292]. Genetic mutations leading to inherited prion diseases can be classified into two distinct groups: (1) abnormal number (addition or deletion) of octapeptide repeats located in the N-terminus of *PRNP* and (2) Point mutations in the C-terminus of *PRNP*. Within the first classification, M129V polymorphism has proven additional importance in determining the age of clinical onset. For example, in 5 or 6 octapeptide repeat insertion (OPRI) mutations, the mean age of clinical onset of disease is about 70 years but an individual heterozygous at residue M129V can have later clinical onset of disease by 10 years as compared to an M/M homozygous individual[293]. Interestingly, an individual homozygous for M/M at residue 129 but has 4-OPRI encounters a later disease onset well into old age and heterozygous individuals do not develop clinical signs of disease; perhaps these individuals outlive the onset of clinical stage disease or they are resistant completely. The second broad classification of inherited prion diseases has a higher prevalence in an infected population. Curiously, some point mutations can be identified by ethno-geographic clustering within a disease phenotype. This is demonstrated in a report by Hsiao et al., where they associated the occurrence of fCJD in Libyan Jews to

a lysine mutation at codon 200[281]. Certain point mutations, coupled with a specific polymorphism at residue 129, create a haplotype that depicts the disease phenotype. Gibbs et al., showed a unique dependence of CJD and FFI phenotypes on the haplotype created at codon 129. If the mutation occurs on the 129 methionine allele, then the patient will develop FFI, but if the patient has the mutation on the 129 valine allele, the patient will almost always present with a CJD phenotype[293]. It is important to note that in a Caucasian population, 43% are methionine homozygotes, 49% are heterozygotes, and 8% are valine homozygotes and in Japan, almost the entire population is methionine homozygous (92%) and little to none are heterozygous at codon 129. Even with the high percentage of M/M individuals in a given population, inherited prion disease is estimated to only affect one case per million or account for just 10% of human disease [294]. A selection of inherited prion diseases have proven experimentally infectious into laboratory animals{Roos:1973vh}. This novel finding excited researchers as this is the first account for a hereditary disease to be shown infectious and substantiated the protein only prion hypothesis. Luckily, epidemiological evidence suggests that familial diseases are not normally infectious within a population, which may account for the minute amount of prion infected individuals.

Sporadic and Infectious Human Diseases

Sporadic CJD (sCJD) accounts for 85% of human prion diseases[295]. Patients that present with clinical signs representative of CJD and do not have known *PRNP* mutations or risk factors are consequently categorized as being inflicted with sporadic CJD. Ultimately the specific cause of sCJD is unknown and cannot be linked to any geographic or temporal clustering[296]. Distinctively, vCJD clinical signs progress within a

few short months (≤6) compared to patients with fCJD[297]. sCJD patients present with either ataxia or cognitive impairment and as the disease progresses those patients that preceded with ataxia will sequentially show signs of cognitive impairment and vice-versa[298] Myoclonus or muscle twitching is a distinct sign that appears as the disease progresses[299]. sCJD trends towards infecting late middle-aged people between 65-79 years of age but becomes fatal in an average of 5 months[290,298].

Prion diseases captured worldwide public attention in the late 1990s when a BSE outbreak crossed the species barrier into humans. In 1996 10 people presented with neurological signs in the UK within months of each other. Surprisingly, the mean onset of disease was 29 years of age while the disease course was sustained an average of 12 months[166]. Additionally, all patients exhibited distinct neuropathology and corresponding clinical signs. Patients primarily presented with behavior changes and progressive dementia but little memory impairment[166]. Spongiform change was prominent throughout the basal ganglia and thalamus while PrP plaques were ubiquitously detected in the cerebrum and cerebellum[166]. Astonishingly, all patients were M/M homozygous at codon 129 and lacked any associated haplotypes of other human prion diseases[166]. In succession with epidemiologic studies, several transmission studies provided unequivocal evidence linking consumption of BSE infected meat with transmission to humans[225,300]. The new disease phenotype was designated as variant CJD (vCJD) because this prion was a new variant to what was previously known for human prion diseases. Since the first outbreak of vCJD, over 200 cases have been identified. This zoonotic transmissible disease not only caused an upheaval in the livestock industry (particularly feed and slaughtering practices) but, in association with

transmissibility of all prion diseases, it has concentrated efforts to minimize iatrogenic transmission in the public.

Iatrogenic transmission of CJD has been well documented as early as in the 1950s[301]. Transmission of CJD through medical procedures has been achieved innocently through corneal grafts,[302]growth hormone therapy,[303] dura mater graphs,[304]multi-use neurosurgical instruments and probes,[305] and the potential of transmission through blood transfusion[250,306,307].Iatrogenic CJD (iCJD) is denoted as sCJD unless sufficient evidence of an iatrogenic event can definitively define otherwise. In most cases, it is impossible to know whether sCJD was transmitted through a medical procedure or blood transfusion because clinical signs are relatively similar. On the rare occasion however, a direct iatrogenic event can be identified proactively. In example, thirteen brain-surgery patients in several states were possibly exposed to sCJD prions through the use of the same contaminated equipment from a possibly sCJD infected patient. The surgical equipment was used on the infected patient for brain surgery just 3 months before death (prion titers in the brain would presumably be high at this end stage of disease) [308]. Because prions are so tolerant of standard sterilization techniques, equipment could have remained contaminated and therefore, transmitted CJD to all naïve patients. Unfortunately, only time (5-10 or more years) will tell as to whether or not individuals develop the invariably fatal disease[308].

Spontaneously Generated Prions

Further evidence that prions are protein-only infectious particles have been demonstrated through spontaneously generated prions *in vitro* using Protein Misfolding Cyclic Amplification (PMCA). PMCA was first utilized by Castilla et al., in 2005 and

revolutionized conventional prion detection methods. PMCA utilizes sonication to break up PK resistant aggregates so the prion seed may ubiquitously interact and template PrP^{C} present in the uninfected brain homogenate substrate[309]. Protocols for PMCA differ across research laboratories but in brief: samples containing a mixture of PrP^{RES} and uninfected brain homogenate go through a 24 hour repetitive cycle of 30 minutes of incubation and 20 seconds of sonication in a water bath maintained at 37^{o} Celsius. Each round is about 144 cycles and at the end of a round, new PrP^{C} substrate is added to the newly generated *in vitro* PrP^{RES} to allow for increased conversion and amplification of the template. This replication technique is analogous to PCR amplifying DNA in a cyclic fashion to detectable amounts. Interestingly, Deleault et al., generated *de novo* PrP^{RES} by altering the PMCA protocol to only include PrP^{C}, co-purified lipids, and polyanions[79,310]. However, skeptics discredit these findings suggesting that cross-contamination cannot be ruled out[76]. Barria and colleagues report that they were able to generate rare *de novo* prions using a modified version of PMCA that consisted of 240 cycles (5 days) and 10 rounds total. Spontaneous generation of a PK resistant prion was only seen in uninfected mouse and hamster brain and not until round 10 using the extended PMCA protocol. These experiments were completed in a prion free laboratory to rule out any chance of cross-contamination. Wang et al., was the first to report their production of an infectious bacterially expressed recombinant prion protein using standard PMCA cycle conditions[311]. John Collinge's group claimed that they inadvertently generated mammalian prions that were distinct from any of their mouse-adapted laboratory strain by binding normal brain homogenate to metal wires[312]. More recently, Zhang and colleagues published that they were able to infect wild-type mice

with one of two *de novo* generated recombinant prions strains propagated using PMCA in a prion free laboratory[313]. Christina Sigurdson's lab added further evidence of the occurrence of spontaneously generated prions with their first report of generating a spontaneous prion disease inadvertently in a mouse model that over-expressed two mutations that confer a rigid loop structure between β2-α2 in the primary PrP sequence, analogous to the loop characterized in the elk prion protein[314]. Even more interestingly, they were able to transmit these spontaneous prions into mice overexpressing the wild-type mouse sequence (TgA20) and sequentially into wild-type mice. It is important to note, however, that clinical onset of disease was observed with a mean incubation of 364 days in TgA20 mice. Though there are no published reports of TgA20 mice being susceptible to spontaneous disease, many unpublished reports and other over-expressing mouse models suggest this as a possible interpretation of late-stage clinical disease. It is also not clear if inoculation of prions into overexpressing mice might accelerate spontaneous disease already "programmed" to occur at a later time[76,315-317]. More convincingly, numerous *in vivo* transgenic mouse models expressing certain *PRNP* mutations have shown the development of spontaneous disease that is transmissible[316,318,319]. The mechanisms that lead to spontaneous generation of prions are controversial and poorly understood.

Are Prions Disguising Themselves as Other Protein Misfolding Diseases?

New theories about prions and their diseases are bridging the gap between other protein misfolding diseases such as Parkinson's and Alzheimer's diseases. Other protein misfolding diseases parallel prion pathogenesis by producing aggregated proteins; this mechanism is implicated in more than 20 human[320,321]. Interestingly, most

aggregated proteins characteristic in other diseases are synonymous to prion structure as they are β-sheet rich oligomers that tend to form amyloid-like aggregates and consequently, are partially protease resistant[322,323]. The solid contradiction between prion diseases and other protein misfolding diseases is the matter of transmissibility. Currently, there is not strong evidence demonstrating transmission of other protein misfolding disease and any studies that suggest the propensity for transmission are full of contradiction. An important challenge or question in transmission studies is incubation time; how long is long enough to determine there was not a transmission event? Baker et al., inoculated primates with an Alzheimer's disease brain homogenate and observed the induction of β-amyloid after 6 years[324]. Furthermore, Kane et al., concluded from their study that β-amyloid was seeded in β-amyloid precursor protein-transgenic mice model infused with an Alzheimer's brain[325]. They did not observe any Aβ-deposition in the brains up to 4 weeks dpi but after 5 months plaques were observable. Meyer-Luehmann published an agreeable paper to this concept also hypothesizing that Aβ-aggregates act as seeds in Alzheimer's pathogenesis[326]. Daringly this group also suggested that there are polymorphic Aβ strains that are reminiscent of the biological activities that prion strains encipher. Other groups have implicated PrP^C as a receptor for Aβ and have concluded that the PrP^C- Aβ interaction suppresses synaptic plasticity and is the cause of memory impairment in Alzheimer disease[327,328]. More recently, You et al., suggested that Aβ neurotoxicity is dependent on triad interaction between PrP^C, copper ions, and N-methyl-D-aspartate receptors[329]. Interactions with PrP^C and other protein misfolding diseases are currently gaining momentum. If a causal link can be identified between all protein misfolding diseases

than more effort and progress can be made to unravel the mechanisms causing neurodegeneration and ultimately, therapeutic strategies could be implemented to help the victims of these fatal, long lasting diseases.

Confounding Prion Names

It is intriguing to observe how the timing and order of prion disease discovery resulted in scrutiny of the protein-only hypothesis. Perhaps viral etiology theories could have been disregarded earlier on if genetic prion diseases were not quickly overshadowed by experimental transmissibility of prion diseases. This could have resulted in an earlier acceptance of the protein-only hypothesis and consequently, prion diseases could have been categorized together earlier and prion disease names could have shown an association. Desperate attempts have been made to umbrella all prion diseases as transmissible spongiform encephalopathies (TSEs) but the common names are primarily used. Acronym names for normal cellular PrP and the misfolded PK resistant form in combination with different strains has only added to the confusion. Prusiner has pleaded for investigators to refer to cellular PrP as PrP^{C}, which has been readily accepted. However, Prusiner has also asked that investigators refer to the misfolded prion as PrP^{SC} even when not referring to scrapie prions. It seems plausible that the infectious form should be reminiscent of the associated disease, i.e., PrP^{SC} should represent scrapie prions and PrP^{CWD} should denote CWD prions and ambiguous prions, not in reference to a specific strain, can be abbreviated as PrP^{RES} to characterize PK resistance.

Much has been discovered about prion diseases since their inception over 200 years ago but their uniqueness and puzzling qualities are perplexing and a generous

amount of experimentation is still required in order to understand prion disease mechanisms fully. The discovery of prion replication and pathological mechanisms may translate to other protein misfolding diseases and lead to therapeutic strategies. Moreover, identification of factors that nascent PrP^{RES} and PrP^{C} utilize in combination with unknown host factors would help elucidate how prion strains are created and ultimately, may lead to the eradication of TSE's forever.

INTRODUCTION TO WORK IN THIS DISSERTATION THESIS

The principle interest in the current research is to gain deeper knowledge about what fundamental factors play a role in prion strain adaptation, to challenge current theories about prion strain fidelity and to assess species barriers and prion strain dynamics with the aid of differential mouse models of prion disease. **The comprehensive hypothesis of this thesis is that host factors, including but not solely PrP^C, mediate prion strain adaptation and determine host range and strength of species barriers.** We employed protein misfolding cyclic amplification (PMCA), transgenic mouse bioassay models expressing variable amounts of PrP^C from mouse and cervid species, and cell culture lines expressing different host PrP^C to navigate through the following questions:

Question 1: Can PMCA be used to mimic *in vivo* strain adaption *in vitro*?

Bioassays of laboratory animals have been the accepted technique for assessing prion transmission susceptibility and ultimately strain detection and characterization since the inception of TSE's. The challenge with studying prion diseases however, has been the dubious incubation periods and the sheer number of animals needed to achieve statistical significance. The development of PMCA has overcome these research barriers by providing an efficient and linear way to replicate prions *in vitro* in a diminutive amount of time. Nonetheless, if PMCA does not mimic *in vivo* mechanisms and outcomes than it is not an appropriate substitute for bioassay. Here we challenge the efficiency and congruency of PMCA by characterizing strain properties of amplified

material in parallel with mouse bioassay by: incubation period, PK resistance, glycoform ratios, lesion profiles, and conformational stability.

Question 2: Are PMCA *de novo* prions infectious and what strain characteristics do they maintain?

The advancement of PMCA as an assay has propelled prion research and further substantiated the protein only hypothesis. PMCA has been scrutinized for its ability to fabricate false positives as a consequence of being highly sensitive (efficient). In most cases, false positives are contributed to laboratory contamination but some reports[76,80,310] indicate that PMCA provides an environment for spontaneous generation of prions *in vitro*. In order to test the rate of false positives in our laboratory, we tested normal brain homogenate substrate using serial protein misfolding cyclic amplification (sPMCA) in a prion free laboratory with new reagents and equipment. To our surprise we were able to generate *de novo* prions within rounds 4, 5, and 7. Spontaneously generated PMCA prions were indistinguishable for cervid prion strains as expected because the normal brain homogenate used as substrate expressed cervid PrP^{C}. We further wanted to test if *de novo* prions were infectious and what strain properties it would emulate. We hypothesized that the PK resistant material generated with PMCA was infectious and transmissible and possess strain properties reminiscent of other cervid prion strains. To test this we inoculated cervid transgenic and wild type mice and subsequently characterized strain properties by incubation period, glycoform ratios, lesion profiles, and conformational stability.

Question 3: Can nascent species barriers be overcome and constructed through prion strain adaptation in a differential host?

A delineating contradiction in the prion research field exists concerning prion strain fidelity and prion strain adaptation. Conflicting data representing both theories have been reported but no clear answer has been established. The ambiguity about prion strains most likely points to a biological overlap or coordination between both theories resulting in both observations. Our lab hypothesizes that PrP^{RES} conformation enciphers prion strain properties by acting as a template for nascent PrP^{RES} but additionally, host factors play a role in adapting prion strains derived from a different host and species barriers can be overcome through this adaptation. To test this hypothesis we inoculated different strains of mice, expressing mouse or cervid PrP^{C}, with different prion strains originating from the same or different host. Additionally, we generated a mouse model that co-over-expresses mouse and cervid PrP^{C} and challenged these mice with different prion strains to assess species barriers and competition between different host-encoded PrP^{C}. Subsequently, we took the strains generated in these mouse models and passaged them into the same or different host to identify species barriers that were overcome or produced.

REFERENCES

1. Perrett, D. From ‘protein’ to the beginnings of clinical proteomics. *Proteomics-Clinical Applications* (2007).
2. Tanford, C. & Reynolds, J. *Nature's robots: a history of proteins*. *2001* (2001).
3. Brock, W. *Justus von Liebig: The chemical gatekeeper*. *2002* (2002).
4. Brock, T. *Milestones in Microbiology: 1546 to 1940*.
5. Koch, R. Die Aetiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des Bacillus anthracis. *Gesammelte Werke von Robert Koch-(Band 1)* (1910).
6. Gordon, S. Elie Metchnikoff: father of natural immunity. *European journal of immunology* (2008).
7. Piro, A., Tagarelli, A. & Tagarelli, G. Paul Ehrlich: the Nobel Prize in physiology or medicine 1908. *International* (2008).
8. Lechevalier, H. Dmitri Iosifovich Ivanovski (1864-1920). *Bacteriological Reviews* **36,** 135 (1972).
9. Beijerinck, M. CONCERNING A CONTAGIUM VIWM FLUIDUM AS CAUSE OF THE SPOT DISEASE OF TOBACCO LEAVES. *1898* 1-22 (1898).
10. Stanley, W. Isolation of a crystalline protein possessing the properties of tobacco-mosaic virus. *Science* (1935).
11. Stanley, W. & Wyckoff, R. The isolation of tobacco ring spot and other virus proteins by ultracentrifugation. *Science* (1937).
12. Stanley, W. VIRUS PROTEINS—A NEW GROUP OF MACROMOLECULES1. *The Journal of Physical Chemistry* (1938).
13. Creutzfeldt, H. Über eine eigenartige herdförmige Erkrankung des Zentralnervensystems (vorläufige Mitteilung). *Zeitschrift für die gesamte Neurologie und Psychiatrie* (1920).
14. Jakob, A. Über eigenartige Erkrankungen des Zentralnervensystems mit bemerkenswertem anatomischen Befunde. *Zeitschrift für die gesamte Neurologie und Psychiatrie* (1921).

15. Plummer, P. J. G. Scrapie—A Disease of Sheep: A Review of the literature. *Canadian Journal of Comparative Medicine and Veterinary Science* **10,** 49 (1946).

16. Fast, C. & Groschup, M. Classical and Atypical Scrapie in Sheep and Goats. *Prions and Diseases* (2013).

17. Gajdusek, D. C. & Zigas, V. Kuru. *The American journal of medicine* **26,** 442-469 (1959).

18. Hershey, A. & Chase, M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *The Journal of general physiology* (1952).

19. Watson, J. & Crick, F. A structure for deoxyribose nucleic acid. *Nature* (1953).

20. Crick, F. & Watson, J. Molecular structure of nucleic acids. *Nature* (1953).

21. Watson, J. & Crick, F. Molecular structure of nucleic acids. *Nature* (1953).

22. Crick, F. & Watson, J. The complementary structure of deoxyribonucleic acid. in *Proceedings of the Royal* (1954).

23. Watson, J. & Crick, F. The structure of DNA. *Cold Spring Harbor Symposia on* (1953).

24. Fraenkel-Conrat, H. Degradation of tobacco mosaic virus with acetic acid. *Virology* **4,** 1-4 (1957).

25. Fraenkel-Conrat, H., Singer, B. & Williams, R. C. Infectivity of viral nucleic acid. *Biochimica et biophysica acta* **25,** 87-96 (1957).

26. Fraenkel-Conrat, H. & Williams, R. C. Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *of Sciences of the United States* (1955).

27. Fraenkel-Conrat, H. THE ROLE OF THE NUCLEIC ACID IN THE RECONSTITUTION OF ACTIVE TOBACCO MOSAIC VIRUS1. *Journal of the American Chemical Society* (1956).

28. Gierer, A. & Schramm, G. Infectivity of ribonucleic acid from tobacco mosaic virus. *1956* (1956).

29. Crick, F. Central dogma of molecular biology. *Nature* (1970).

30. Sigurdsson, B. Rida, a chronic encephalitis of sheep. With general remarks on infections, which develop slowly, and some of their special characteristics. *Br Vet J 1954* 341–354

31. Hadlow, W. J. Scrapie and kuru. *The Lancet* **274,** 289-290

32. KLATZO, I., GAJDUSEK, D. C. & ZIGAS, V. Pathology of Kuru. *Laboratory nvestigation; a journal of technical methods and pathology* **8,** 799-847 (1959).

33. Alpers, M. P., Asher, D., Gajdusek, D. C. & Gibbs, C. Creutzfeldt-Jakob disease The neuropathology of a transmission experiment. *Brain* (1969).

34. Gajdusek, D. C., Gibbs, C. & Alpers, M. Transmission and passage of experimental 'kuru'to chimpanzees. *Science* (1967).

35. GAJDUSEK, D., Clarence, J., Jr & ALPERS, M. Slow, latent, and temperate virus infections. *Slow* (1965).

36. GORDON, W. S. Advances in veterinary research. *The Veterinary record* **58,** 516-25 (1946).

37. Alper, T. *et al.* Does the Agent of Scrapie Replicate without Nucleic Acid ? *Nature* **214,** 764-766 (1967).

38. Alper, T., Haig, D. A. & Clarke, M. C. The exceptionally small size of the scrapie agent. *Biochemical and biophysical research communications* **22,** 278-84 (1966).

39. Bellinger-Kawahara, C., Cleaver, J. E., Diener, T. O. & Prusiner, S. B. Purified scrapie prions resist inactivation by UV irradiation. *Journal of virology* **61,** 159-66 (1987).

40. Latarjet, R., Muel, B., Da H & CLARKE, T. M. Inactivation of the scrapie agent by near monochromatic ultraviolet light. *1970* (1970).

41. Griffith, J. S. Self-replication and scrapie. *Nature* **215,** 1043-4 (1967).

42. HUNTER, G. D. & MILLSON, G. C. STUDIES ON THE HEAT STABILITY AND CHROMATOGRAPHIC BEHAVIOUR OF THE SCRAPIE AGENT. *Journal of general microbiology* **37,** 251-8 (1964).

43. PATTISON, I. H. RESISTANCE OF THE SCRAPIE AGENT TO FORMALIN. *Journal of comparative pathology* **75,** 159-64 (1965).

44. CHANDLER, R. L. Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* **1,** 1378-9 (1961).

45. Meggendorfer, F. Klinische und genealogische Beobachtungen bei einem Fall von spastischer Pseudosklerose Jakobs. *Zeitschrift für die gesamte Neurologie und Psychiatrie* (1930).

46. Masters, C., Harris, J. & Gajdusek, D. C. Creutzfeldt-Jakob disease: Patterns of worldwide occurrence and the significance of familial and sporadic clustering. *Annals of* (1979).

47. Masters, C. & Gibbs, C., Jr. Transmissible spongiform encephalopathy (Creutzfeldt-Jakob disease). Atypical clinical and pathological findings. *Archives of* (1981).

48. Chandler, R. L. Experimental scrapie in the mouse. *Res. Vet. Sci* **4,** 276 (1963).

49. Eklund, C. & Hadlow, W. Some properties of the scrapie agent and its behavior in mice. in *Proceedings of the Society* (1963).

50. Experimental transmission of scrapie to golden hamsters. *The Lancet* **282,** 1072 (1963).

51. Kimberlin, R. H. & Walker, C. A. Characteristics of a short incubation model of scrapie in the golden hamster. *Journal of General Virology* (1977).

52. Kimberlin, R. H. & Marsh, R. Comparison of scrapie and transmissible mink encephalopathy in hamsters. I. Biochemical studies of brain during development of disease. *Journal of Infectious Diseases* (1975).

53. Kimberlin, R. H. & Walker, C. A. Evidence that the Transmission of One Source of Scrapie Agent to Hamsters Involves Separation of Agent Strains from a Mixture. *Journal of General Virology* **39,** 487-496 (1978).

54. Prusiner, S. B. An approach to the isolation of biological particles using sedimentation analysis. *The Journal of biological chemistry* **253,** 916-21 (1978).

55. Pattison, I. H. & Jones, K. M. The possible nature of the transmissible agent of scrapie. *The Veterinary record* **80,** 2-9 (1967).

56. Hunter, G. D., Gibbons, R. A., Kimberlin, R. H. & Millson, G. C. Further studies of the infectivity and stability of extracts and homogenates derived from scrapie affected mouse brains. *Journal of comparative pathology* **79,** 101-8 (1969).

57. Cho, H. J. Requirement of a protein component for scrapie infectivity. *Intervirology* **14,** 213-6 (1980).

58. Prusiner, S. B. Novel proteinaceous infectious particles cause scrapie. *Science* **216,** 136-144 (1982).

59. McKinley, M. & Bolton, D. A protease-resistant protein is a structural component of the scrapie prion. *Cell* (1983).

60. DIRINGER, H., HILMERT, H. & SIMON, D. Towards purification of the scrapie agent. *European Journal of* (1983).

61. Bolton, D. & McKinley, M. Identification of a protein that purifies with the scrapie prion. *Science* (1982).

62. Prusiner, S. B. *et al.* Electrophoretic properties of the scrapie agent in agarose gels. *Proceedings of the National Academy of Sciences of the United States of America* **77,** 2984-8 (1980).

63. Prusiner, S. B. *et al.* Further purification and characterization of scrapie prions. *Biochemistry* **21,** 6942-50 (1982).

64. Prusiner, S. B. *et al.* Partial purification and evidence for multiple molecular forms of the scrapie agent. *Biochemistry* **17,** 4993-9 (1978).

65. Prusiner, S. B. *et al.* Measurement of the scrapie agent using an incubation time interval assay. *Annals of neurology* **11,** 353-8 (1982).

66. Büeler, H. *et al.* Mice devoid of PrP are resistant to scrapie. *Cell* **73,** 1339-1347 (1993).

67. Raeberi, A., Fischert, M., Saileri, A. & Kobayashit, Y. Normal host prion protein necessary for scrapie-induced neurotoxicity. 1-5 (1996).

68. Groth, D. & Serban, A. Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. in *Proceedings of the* (1993).

69. Circumventing tolerance to generate autologous monoclonal antibodies to the prion protein. in *Proceedings of the* (1996).

70. Atarashi, R. *et al.* Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. *Nature methods* **5,** 211-2 (2008).

71. Caughey, B., Kocisko, D. A., Raymond, G. J. & Lansbury, P. T., Jr. Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. *Chemistry & biology* **2,** 807-17 (1995).

72. Castilla, J. *et al.* Protein misfolding cyclic amplification for diagnosis and prion propagation studies. *Methods in enzymology* **412,** 3-21 (2006).

73. Saborio, G., Permanne, B. & Soto, C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* (2001).

74. Meyerett, C. *et al.* In vitro strain adaptation of CWD prions by serial protein misfolding cyclic amplification. *Virology* **382,** 267-276 (2008).

75. Soto, C. Prion hypothesis: the end of the controversy? *Trends in biochemical sciences* (2011).

76. Barria, M. A., Mukherjee, A., Gonzalez-Romero, D., Morales, R. & Soto, C. De Novo Generation of Infectious Prions In Vitro Produces a New Disease Phenotype. *PLoS Pathogens* **5,** e1000421 (2009).

77. Soto, C. & Castilla, J. The controversial protein-only hypothesis of prion propagation. *2004* (2004).

78. Castilla, J. & Soto, C. Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. *Journal of Biological Chemistry* (2006).

79. Deleault, N. R., Geoghegan, J. & Nishina, K. Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. *Journal of Biological* (2005).

80. Deleault, N. R. & Lucassen, R. W. RNA molecules stimulate prion protein conversion. *Nature* **425,** 717-720 (2003).

81. Brown, J. H. A. A. P. *et al.* Neuropathological Diagnostic Criteria for Creutzfeldt&###x2010;Jakob Disease (CJD) and Other Human Spongiform Encephalopathies (Prion Diseases). *Brain Pathology* **5,** 459-466 (1995).

82. Prusiner, S. B. Prion Diseases and the BSE Crisis. *Science* **278,** 245-251

83. Calvi, C. *et al.* Characterization of Conformation-dependent Prion Protein Epitopes. *Journal of Biological Chemistry* **287,** 37219-37232 (2012).

84.Da Harris, Huber, M., van Dijken, P. & Shyng, S. Processing of a cellular prion protein: identification of N-and C-terminal cleavage sites. *Biochemistry* (1993).

85. Cobb, N. & Surewicz, W. Prion diseases and their biochemical mechanisms†. *Biochemistry* (2009).

86. Caughey, B., Race, R., Ernst, D. & Buchmeier, M. Prion protein biosynthesis in scrapie-infected and uninfected neuroblastoma cells. *Journal of* (1989).

87. Pan, K., Baldwin, M. & Nguyen, J. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. in *Proceedings of the* (1993).

88. Riek, R., Hornemann, S., Wider, G. & Billeter, M. NMR structure of the mouse prion protein domain PrP (121-231). *Nature* (1996).

89. Liu, H., Farr-Jones, S., Ulyanov, N. & Llinas, M. Solution structure of Syrian hamster prion protein rPrP (90-231). *Biochemistry* (1999).

90. Tempst, P., Teplow, D., Le Hood & Prusiner, S. B. A cellular gene encodes scrapie PrP 27-30 protein. *Cell* (1985).

91. Gossert, A., Bonjour, S. & Da Lysek. Prion protein NMR structures of elk and of mouse/elk hybrids. in *Proceedings of the* (2005).

92. Gorfe, A. A. & Caflisch, A. Ser170 controls the conformational multiplicity of the loop 166–175 in prion proteins: implication for conversion and species barrier. *The FASEB journal* 3279-3287 (2007).

93. Riek, R., Hornemann, S., Wider, G. & Glockshuber, R. NMR characterization of the full-length recombinant murine prion protein, m PrP (23–231). *FEBS letters* (1997).

94. Zahn, R., Liu, A., Lührs, T. & Riek, R. NMR solution structure of the human prion protein. in *Proceedings of the* (2000).

95. Hornshaw, M., McDermott, J., C, J., Candy, J. & y. Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. *and biophysical research* (1995).

96. Garnett, A. & Viles, J. Copper Binding to the Octarepeats of the Prion Protein Affinity, Specificity, Folding, and Cooperativity: Insights from Circular Dichroism. *Journal of Biological Chemistry* (2003).

97. Jackson, G., Murray, I. & Hosszu, L. Location and properties of metal-binding sites on the human prion protein. in *Proceedings of the* (2001).

98. Requena, J. Copper-catalyzed oxidation of the recombinant SHa (29–231) prion protein. in *Proceedings of the* (2001).

99. Ford, M., Burton, L., Morris, R. & Hall, S. Selective expression of prion protein in peripheral tissues of the adult mouse. *Neuroscience* (2002).

100. Angers, R. C. *et al.* Chronic Wasting Disease Prions in Elk Antler Velvet. *Emerging Infectious Diseases* **15,** 696-703 (2009).

101. Browning, S. R. & Sigurdson, C. J. Prions in skeletal muscles of deer with chronic wasting disease. *Science* (2006).

102. Bendheim, P., Brown, H., Rudelli, R. & Scala, L. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology* (1992).

103. Fischer, M., Rülicke, T., Raeber, A. & Sailer, A. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *The EMBO* (1996).

104. Bluethmann, H., Lipp, H. & DeArmond, S. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *1992* (1992).

105. Brandner, S., ner, Raeber, A. & Sailer, A. Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system. in *Proceedings of the* (1996).

106. Chiarini, L., Freitas, A. & Zanata, S. Cellular prion protein transduces neuroprotective signals. *The EMBO* 1-10 (2002).

107. Lopes, M., Hajj, G. & Muras, A. Interaction of cellular prion and stress-inducible protein 1 promotes neuritogenesis and neuroprotection by distinct signaling pathways. *The Journal of* 1-10 (2005).

108. Brown, D. & BESINGER, A. Prion protein expression and superoxide dismutase activity. *Biochem J* (1998).

109. Kim, B., Lee, H., Choi, J., Kim, J. & Choi, E. The cellular prion protein (PrP C) prevents apoptotic neuronal cell death and mitochondrial dysfunction induced by serum deprivation. *Molecular brain* (2004).

110. Bounhar, Y., Goodyer, C. & LeBlanc, A. Prion protein protects human neurons against Bax-mediated apoptosis. *Journal of Biological* (2001).

111. Brown & Nicholas, R. Lack of prion protein expression results in a neuronal phenotype sensitive to stress. *Journal of neuroscience* 1-14 (2002).

112. Wong, B., Pan, T., Liu, T. & Li, R. Differential Contribution of Superoxide Dismutase Activity by Prion Protein in Vivo. *and biophysical research* 1-4 (2000).

113. Wopfner, F., Weidenhöfer, G. & Schneider, R. Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *Journal of molecular* (1999).

114. Rosenthal, N., Keesey, J., Cr, B., Crandall, B. & all. Familial neurological disease associated with spongiform encephalopathy. *Archives of* (1976).

115. Stender, A. Weitere Beiträge zum Kapitel „Spastische Pseudosklerose Jakobs". *Zeitschrift für die gesamte Neurologie und Psychiatrie* (1930).

116. Hsiao, K., Baker, H., Crow, T. J., Poulter, M. & Owen, F. Linkage of a prion protein missense variant to Gerstmann-Str~ iussler syndrome. *Nature* (1989).

117. Hsiao, K. & Baker, H. Linkage of a prion protein missense variant to Gerstmann–Sträussler syndrome. (1989).

118. Palmer, M., Dryden, A., Hughes, J. & Collinge, J. Homozygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease. *1991* (1991).

119. Goldmann, W., Hunter, N., Benson, G., Foster, J. & Hope, J. Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene. *J Gen Virol* (1991).

120. Westaway, D., Zuliani, V. & Cooper, C. Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie. *Genes &* (1994).

121. Basler, K., Oesch, B., Scott, M., Westaway, D. & Wälchli, M. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* (1986).

122. Manson, J., West, J., Thomson, V. & McBride, P. The prion protein gene: a role in mouse embryogenesis? (1992).

123. Moser, M., Colello, R., Pott, U. & Oesch, B. Developmental expression of the prion protein gene in glial cells. *Neuron* (1995).

124. Mobley, W. & Neve, R. Nerve growth factor increases mRNA levels for the prion protein and the beta-amyloid protein precursor in developing hamster brain. in *Proceedings of the* (1988).

125. Moore, R. C. & Westaway, D. Two different neurodegenerative diseases caused by proteins with similar structures. in *Proceedings of the* (2001).

126. Moore, R. C., Lee, I., Silverman, G. & Harrison, P. Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *Journal of molecular* (1999).

127. Behrens, A., Genoud, N., Naumann, H. & Rülicke, T. Absence of the prion protein homologue Doppel causes male sterility. *The EMBO* (2002).

128. McConnell, I. *et al.* The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. *The Journal of general virology* **72 (Pt 3),** 595-603 (1991).

129. Carlson, G., Kingsbury, D., Goodman, P. & Coleman, S. Linkage of prion protein and scrapie incubation time genes. *Cell* (1986).

130. Moore, R. C. *et al.* Mice with gene targetted prion protein alterations show that Prnp, Sine and Prni are congruent. *Nature genetics* **18,** 118-125 (1998).

131. Parry, H. Scrapie: a transmissible hereditary disease of sheep. *Nature* (1960).

132. Vey, M. & Pilkuhn, S. COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. in *Proceedings of the* (1997).

133. Zulianello, L., Erpel, S. & Han, D. Dominant-negative inhibition of prion formation diminished by deletion mutagenesis of the prion protein. *Journal of* (2000).

134. Perrier, V. & Safar, J. Dominant-negative inhibition of prion replication in transgenic mice. in *Proceedings of the* (2002).

135. Goldmann, W., Smith, G. & Foster, J. PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. *Journal of General* (1994).

136. Goldmann, W., Martin, T. & Foster, J. Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period. *Journal of General* (1996).

137. Obermaier, G., Kretzschmar, H. & Hafner, A. Spongiform central nervous system myelinopathy in African dwarf goats. *Journal of comparative* (1995).

138. Goldmann, W. & Gravenor, M. Epidemiological implications of the susceptibility to BSE of putatively resistant sheep. *Journal of General* (2003).

139. Houston, F., Goldmann, W., Chong, A. & Jeffrey, M. Prion diseases: BSE in sheep bred for resistance to infection. *Nature* (2003).

140. Green, K. M., Browning, S. R. & Seward, T. S. The elk PRNP codon 132 polymorphism controls cervid and scrapie prion propagation. *Journal of General* 598-608 (2008).

141. Palmer, M. *Prion diseases. 1997* (1997).

142. Wadsworth, J., Asante, E. & Desbruslais, M. Human prion protein with valine 129 prevents expression of variant CJD phenotype. *Science* (2004).

143. Manson, J. C. *et al.* 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Molecular Neurobiology* **8,** 121-127 (1994).

144. Mallucci, G., Ratte, S., Asante, E. & Linehan, J. Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *The EMBO* (2002).

145. Sakaguchi, S. *et al.* Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. *, Published online: 11 April 1996;* | *doi:10.1038/380528a0* **380,** 528-531 (1996).

146. Torchia, M., Cohen, F. E., DeArmond, S. & Prusiner, S. B. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* (1995).

147. Telling, G. C., Scott, M. & Hsiao, K. Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. in *Proceedings of the* (1994).

148. Prusiner, S. B. *et al.* Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63,** 673-86 (1990).

149. Browning, S. R. *et al.* Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *Journal of* 13345-13350 (2004).

150. Pattison, I. H., Gordon, W. S. & Millson, G. C. Experimental Production of Scrapie in Goats. *Journal of Comparative Pathology and Therapeutics* **69,** 300-IN20 (1959).

151. Marsh, R. *et al.* Epidemiological and experimental studies on a new incident of transmissible mink encephalopathy. *The Journal of general virology* **72 (Pt 3),** 589-594 (1991).

152. Wilson, D. R., Anderson, R. D. & Smith, W. Studies in scrapie. *Journal of Comparative Pathology and Therapeutics* **60,** 267-IN12 (1950).

153. Zlotnik, I. & Rennie, J. C. The pathology of the brain of mice inoculated with tissues from scrapie sheep. *Journal of Comparative Pathology and Therapeutics* **72,** 360-IN44 (1962).

154. CHANDLER, R. L. & FISHER, J. EXPERIMENTAL TRANSMISSION OF SCRAPIE TO RATS. *Lancet* **2,** 1165 (1963).

155. Zlotnik, I. & Rennie, J. C. Further observations on the experimental transmission of scrapie from sheep and goats to laboratory mice. *Journal of Comparative Pathology and Therapeutics* **73,** 150-IN13 (1963).

156. WILLIAMS, E. S., WILLIAMS, E. S., Young, S. & YOUNG, S. CHRONIC WASTING DISEASE OF CAPTIVE MULE DEER: A SPONGIFORM ENCEPHALOPATHY. *Journal of wildlife diseases* **16,** 89-98 (1980).

157. Fraser, H. The sequential development of the brain lesions of scrapie in three strains of mice. *Journal of comparative pathology* (1968).

158. Bruce, M. & Dickinson, A. G. Cerebral amyloidosis in scrapie in the mouse: effect of agent strain and mouse genotype. *and Applied Neurobiology* (1976).

159. Williams, E. S., Johnson, F. & Young, S. Amyloid plaques in spongiform encephalopathy of mule deer. *Journal of comparative* (1985).

160. Williams, E. S. & Young, S. SPONGIFORM ENCEPHALOPATHY OF ROCKY MOUNTAIN ELK 1. *Journal of wildlife diseases* (1982).

161. DeArmond, S., Mobley, W., DeMott, D. & Barry, R. Changes in the localization of brain prion proteins during scrapie infection. *Neurology* (1987).

162. Bruce, M. Genetic control of amyloid plaque production and incubation period in scrapie-infected mice. *Journal of Neuropathology &* (1985).

163. Wilesmith, J. & Wells, G. Bovine spongiform encephalopathy. *Transmissible Spongiform Encephalopathies:* (1991).

164. Bessen, R. *et al.* Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters. *The Journal of general virology* **73 (Pt 2),** 329-334 (1992).

165. Sidle, K. C. L., Meads, J., Ironside, J., Hill, A. F. & Ironside, A. F. J. M. J. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *, Published online: 24 October 1996; | doi:10.1038/383685a0* **383,** 685-690 (1996).

166. Will, R. G. *et al.* A new variant of Creutzfeldt-Jakob disease in the UK. *The Lancet* **347,** 921-925 (1996).

167. Prusiner, S. B. & Scott, M. R. Genetics of prions. *Annual review of genetics* **31,** 139-75 (1997).

168. Safar, J. *et al.* Eight prion strains have PrPSc molecules with different conformations. *Nature medicine* (1998).

169. Kuczius, T. & Groschup, M. Differences in proteinase K resistance and neuronal deposition of abnormal prion proteins characterize bovine spongiform encephalopathy (BSE) and scrapie strains. *Molecular Medicine* (1999).

170. Bartz, J., Bessen, R., McKenzie, D. & Marsh, R. Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. *Journal of* (2000).

171. Peretz, D., Williamson, R. A., Legname, G. & Matsunaga, Y. A change in the conformation of prions accompanies the emergence of a new prion strain. *Neuron* 921-932 (2002).

172. Asante, E., Linehan, J., Desbruslais, M. & Joiner, S. BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *The EMBO* 1-9 (2002).

173. Sigurdson, C. J. *et al.* Strain Fidelity of Chronic Wasting Disease upon Murine Adaptation. *Journal of Virology* **80,** 12303-12311 (2006).

174. Tamgüney, G. *et al.* Chimeric elk/mouse prion proteins in transgenic mice. *Journal of General Virology* **94,** 443-452 (2013).

175. Goldmann, W. & Foster, J. Natural scrapie and PrP genotype: case-control studies in British sheep. *Veterinary* (1997).

176. Detwiler, L. & Baylis, M. The epidemiology of scrapie. *Revue Scientifique et* (2003).

177. Amigues, Y., Schelcher, F., Ducrocq, V. & Andreoletti, O. Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. *Archives of* (1999).

178. Jeffrey, M., Martin, S., Gonzalez, L. & Ryder, S. Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. *Journal of comparative* (2001).

179. Benestad, S., Arsac, J., Goldmann, W. & Nöremark, M. Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology. *Veterinary research* (2008).

180. Lühken, G., Groschup, M. & Erhardt, G. Prion protein allele A136 H 154 Q 171 is associated with high susceptibility to scrapie in purebred and crossbred German Merinoland sheep. *Archives of virology* (2004).

181. Lühken, G., Buschmann, A., Brandt, H. & Eiden, M. Epidemiological and genetical differences between classical and atypical scrapie cases. *Veterinary* (2007).

182. Moreno, C., Moazami-Goudarzi, K. & Laurent, P. Which PrP haplotypes in a French sheep population are the most susceptible to atypical scrapie? *Archives of* (2007).

183. Benestad, S., Sarradin, P. & Thu, B. Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Veterinary* (2003).

184. Lühken, G. & Schultz, J. Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR). *Journal of General* (2004).

185. Nentwig, A. *et al.* Diversity in neuroanatomical distribution of abnormal prion protein in atypical scrapie. *PLoS Pathogens* **3,** e82 (2007).

186. Vidal, E. *et al.* Lack of PrP(sc) immunostaining in intracranial ectopic lymphoid follicles in a sheep with concomitant non-suppurative encephalitis and Nor98-like atypical scrapie: a case report. *Veterinary journal (London, England : 1997)* **177,** 283-8 (2008).

187. Andréoletti, O., Orge, L., Benestad, S. & Beringue, V. Atypical/Nor98 scrapie infectivity in sheep peripheral tissues. *PLoS* (2011).

188. Hoinville, L. A review of the epidemiology of scrapie in sheep. *Revue scientifique et technique (International Office of* (1996).

189. Onodera, T., Ikeda, T. & Muramatsu, Y. Isolation of scrapie agent from the placenta of sheep with natural scrapie in Japan. *Microbiology and* (1993).

190. Hadlow, W. & Kennedy, R. Natural infection of Suffolk sheep with scrapie virus. *Journal of Infectious* (1982).

191. Hagenaars, T. The transmission dynamics of the aetiological agent of scrapie in a sheep flock. *Mathematical* (2000).

192. Dickinson, A. G., Stamp, J. T. & Renwick, C. C. Maternal and lateral transmission of scrapie in sheep. *Journal of comparative pathology* 19-25 (1974).

193. O'Rourke, K., Holyoak, G. & Clark, W. PrP genotypes and experimental scrapie in orally inoculated Suffolk sheep in the United States. *Journal of General* (1997).

194. Goldmann, W. & Benson, G. … sheep affected by natural scrapie differ significantly in PrP genotype frequencies from healthy sheep and those selected for reduced incidence of scrapie. *Journal of General* (1993).

195. Pattison, I. H. Scrapie—a personal view. *Journal of Clinical Pathology* 1-6 (1972).

196. Foster, J., Goldmann, W., Stear, M. & Hope, J. Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Archives of* (1996).

197. Foster, J. & Williams, A. Observations on the transmission of scrapie in experiments using embryo transfer. *Veterinary* (1996).

198. Foster, J. *et al.* Studies on maternal transmission of scrapie in sheep by embryo transfer. *Veterinary Record* **130,** 341-343 (1992).

199. Brotherston, J., Renwick, C. C., Stamp, J. T. & Zlotnik, I. Spread of scrapie by contact to goats and sheep. *Journal of comparative* (1968).

200. Ryder, S., Dexter, G., Bellworthy, S. & Tongue, S. Demonstration of lateral transmission of scrapie between sheep kept under natural conditions using lymphoid tissue biopsy. *Research in veterinary science* (2004).

201. PALSSON, P. RIDA (SCRAPIE) IN ICELANDIC SHEEP AND ITS EPIDEMIOLOGY. *1980* (1980).

202. Ryder, S., Spencer, Y. & Bellerby, P. Immunohistochemical detection of PrP in the medulla oblongata of sheep: the spectrum of staining in normal and scrapie-affected sheep. *Veterinary* (2001).

203. Jendroska, K., Heinzel, F., Torchia, M. & Stowring, L. Proteinase-resistant prion protein accumulation in Syrian hamster brain correlates with regional pathology and scrapie infectivity. *Neurology* (1991).

204. Lasmézas, C., Deslys, J. & Demaimay, R. Strain specific and common pathogenic events in murine models of scrapie and bovine spongiform encephalopathy. *Journal of General* 1-9 (1996).

205. Unknown. CLINICAL SIGNS OF BOVINE SPONGIFORM ENCEPHALOPATHY IN CATTLE. *http://www.defra.gov.uk/ahvla-en/files/clinical-signs-bse-cattle-stills.pdf*

206. Anderson, R. M. *et al.* Transmission dynamics and epidemiology of BSE in British cattle. *Nature* **382,** 779-788 (1996).

207. Prusiner, S. B. *et al.* Immunologic and molecular biologic studies of prion proteins in bovine spongiform encephalopathy. *The Journal of infectious diseases* **167,** 602-13 (1993).

208. Goldmann, W. & Smith, G. Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. *Veterinary* (1994).

209. Goldmann, W., Martin, T., Dawson, M. & Hope, J. Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon. *The Journal of general virology* **72 (Pt 1),** 201-4 (1991).

210. Biacabe, A. -., Laplanche, J., Ryder, S. & Baron, T. Distinct molecular phenotypes in bovine prion diseases. *EMBO reports* (2004).

211. Casalone, C., Zanusso, G. & Acutis, P. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. in *Proceedings of the* (2004).

212. Yamakawa, Y., Hagiwara, K. & Nohtomi, K. Atypical Proteinase K-Resistant Prion Protein (PrP^ r^ e^ s) Observed in an Apparently Healthy 23-Month-Old Holstein Steer. *Japanese journal of* (2003).

213. de Bosschere, H. & Roels, S. Atypical case of bovine spongiform encephalopathy in an East-Flemish cow in Belgium. *Journal of* (2004).

214. Buschmann, A. *et al.* Atypical BSE in Germany—Proof of transmissibility and biochemical characterization. *Veterinary* **117,** 103-116 (2006).

215. Schreuder, B. General aspects of transmissible spongiform encephalopathies and hypotheses about the agents. *Veterinary Quarterly* (1993).

216. Taylor, K. Origin of BSE. *The Veterinary record* **137,** 674-5 (1995).

217. Wilesmith, J., Ryan, J. & Atkinson, M. Bovine spongiform encephalopathy: epidemiological studies on the origin. *Veterinary Record* (1991).

218. Wilesmith, J., Wells, G. & Cranwell, M. Bovine spongiform encephalopathy: epidemiological studies. *Veterinary* (1988).

219. Doherr, M. Bovine Spongiform Encephalopathy (BSE)–Infectious, Contagious, Zoonotic or Production. *Acta vet scand* (2003).

220. Kamphues, J. [Alternatives to presently established forms of animal body removal--tolerated, intended and feared?]. *DTW Deutsche tierarztliche Wochenschrift* (1997).

221. Paisley, L. & Hostrup-Pedersen, J. A quantitative assessment of the risk of transmission of bovine spongiform encephalopathy by tallow-based calf milk-replacer. *Preventive veterinary medicine* (2004).

222. Eddy, R. G. Origin of BSE. *The Veterinary record* **137,** 648 (1995).

223. Fraser, H., Bruce, M., Chree, A., Mcconnell, I. & Wells, G. Transmission of bovine spongiform encephalopathy to mice. *Veterinary Record* (1988).

224. Dawson, M., Wells, G. & Parker, B. Preliminary evidence of the experimental transmissibility of bovine spongiform encephalopathy to cattle. *Veterinary Record* (1990).

225. Bruce, M., Will, R. G., Ironside, J. & McConnell, I. Transmissions to mice indicate that ‘new variant’CJD is caused by the BSE agent. *Nature* (1997).

226. Hadlow, W., Huff, T. & Wells, G. Experimental infection of mink with bovine spongiform encephalopathy. *Journal of General* (1994).

227. Baxa, U., Taylor, K., Wall, J., Simon, M. & Cheng, N. Architecture of Ure2p Prion Filaments THE N-TERMINAL DOMAINS FORM A CENTRAL CORE FIBER. *Journal of Biological* (2003).

228. Kirkwood, J., Cunningham, A. & Flach, E. Spongiform encephalopathy in another captive cheetah (Acinonyx jubatus): evidence for variation in susceptibility or incubation periods between species? *Journal of Zoo and* (1995).

229. Wilesmith, J. Bovine spongiform encephalopathy. *of the New York Academy of* (1994).

230. Cooper, J. & Bird, S. UK bovine carcass meat consumed as burgers, sausages and other meat products: by birth cohort and gender. *Journal of cancer epidemiology and* (2002).

231. Ghetti, B., Beavis, R., Bugiani, O. & Frangione, B. … prion protein (PrP) fragment, an integral component of the PrP region required for infectivity, is the major amyloid protein in Gerstmann-Sträussler-Scheinker disease …. *Journal of Biological* (2001).

232. Middleton, D. & Barlow, R. Failure to transmit bovine spongiform encephalopathy to mice by feeding them with extraneural tissues of affected cattle. *Veterinary Record* (1993).

233. Wells, G. A. *et al.* Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy. *The Veterinary record* **135,** 40-1 (1994).

234. Wells, G. A. *et al.* Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *The Veterinary record* **142,** 103-6 (1998).

235. Williams, E. S. Chronic wasting disease. *Veterinary Pathology Online* 530-549 (2005).

236. Miller, M. W. *et al.* Lions and Prions and Deer Demise. *PLoS One* **3,** e4019 (2008).

237. Jewell, J. E., Conner, M. & Wolfe, L. Low frequency of PrP genotype 225SF among free-ranging mule deer (Odocoileus hemionus) with chronic wasting disease. *Journal of General Virology* **86,** 2127-2134 (2005).

238. Johnson, C., Johnson, J., JP, V, Vanderloo, J. & erloo. Prion protein polymorphisms in white-tailed deer influence susceptibility to chronic wasting disease. *Journal of General Virology* **87,** 2109-2114 (2006).

239. O'Rourke, K., Spraker, T. & Hamburg, L. Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. *Journal of General* (2004).

240. Hamir, A., Gidlewski, T. & Spraker, T. Preliminary observations of genetic susceptibility of elk (Cervus elaphus nelsoni) to chronic wasting disease by experimental oral inoculation. *Journal of Veterinary* (2006).

241. O'Rourke, K., Spraker, T., Zhuang, D. & Greenlee, J. Elk with a long incubation prion disease phenotype have a unique PrPd profile. (2007).

242. Williams, E. S., Williams, E. S., Williams, E. S., Young, S. & YOUNG, S. CHRONIC WASTING DISEASE OF CAPTIVE MULE DEER: A SPONGIFORM ENCEPHALOPATHY. *Journal of wildlife diseases* **16,** 89-98 (1980).

243. Williams, E. S. & Young, S. Spongiform encephalopathies in Cervidae. *Revue scientifique et technique (* (1992).

244. Hamir, A., Miller, J., Cutlip, R. & Kunkle, R. Transmission of sheep scrapie to elk (Cervus elaphus nelsoni) by intracerebral inoculation: final outcome of the experiment. *Journal of Veterinary* (2004).

245. Hamir, A., Kunkle, R., Cutlip, R. & Miller, J. Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. *Journal of Veterinary* (2005).

246. Hamir, A., Miller, J., Kunkle, R. & Hall, S. Susceptibility of cattle to first-passage intracerebral inoculation with chronic wasting disease agent from white-tailed deer. *Veterinary Pathology* (2007).

247. Hamir, A., Kunkle, R., Cutlip, R. & Miller, J. Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation. *Journal of Veterinary* (2006).

248. Spraker, T., Miller, M. W. & Sigurdson, C. J. Infectious prions in the saliva and blood of deer with chronic wasting disease. *Science* (2006).

249. Mathiason, C., Carver, S., Zabel, M. & Telling, G. C. Detection of chronic wasting disease prions in salivary, urinary, and intestinal tissues of deer: potential mechanisms of prion shedding and transmission. *Journal of* (2011).

250. Mathiason, C., Hays, S., Powers, J. & Hayes-Klug, J. Infectious prions in pre-clinical deer and transmission of chronic wasting disease solely by environmental exposure. *PLoS One* (2009).

251. Haley, N., Seelig, D. & Zabel, M. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. *PLoS One* (2009).

252. Sigurdson, C. J. A prion disease of cervids: Chronic wasting disease. *Veterinary research* **39,** 41 (2008).

253. Nichols, T. *et al.* Detection of protease-resistant cervid prion protein in water from a CWD-endemic area. *Prion* **3,** 171

254. Wyckoff, A. C., Lockwood, K., Meyerett-Reid, C. & Michel, B. Estimating Prion Adsorption Capacity of Soil by BioAssay of Subtracted Infectivity from Complex Solutions (BASICS). *PLoS One* (2013).

255. Tamgüney, G., Miller, M. W., Wolfe, L. & Sirochman, T. Asymptomatic deer excrete infectious prions in faeces. *Nature* (2010).

256. Pulford, B., Spraker, T. & Wyckoff, A. C. Detection of PrPCWD in feces from naturally exposed Rocky Mountain elk (Cervus elaphus nelsoni) using protein misfolding cyclic amplification. *Journal of Wildlife* (2012).

257. Wolfe, L., Spraker, T. & González, L. PrPCWD in rectal lymphoid tissue of deer (Odocoileus spp.). *Journal of General* (2007).

258. Spraker, T., VerCauteren, K. & Gidlewski, T. Antemortem Detection of PrPCWD in Preclinical, Ranch-Raised Rocky Mountain Elk (Cevvus Elaphus Nelsoni) by Biopsy of the Rectal Mucosa. *Journal of Veterinary* (2009).

259. Spraker, T. & Gidlewski, T. Detection of PrPCWD in postmortem rectal lymphoid tissues in Rocky Mountain elk (Cervus elaphus nelsoni) infected with chronic wasting disease. *Journal of Veterinary* (2006).

260. Haley, N., Mathiason, C. & Zabel, M. Detection of sub-clinical CWD infection in conventional test-negative deer long after oral exposure to urine and feces from CWD+ deer. *PLoS One* (2009).

261. Race, R., Raines, A., Baron, T. & Miller, M. Comparison of abnormal prion protein glycoform patterns from transmissible spongiform encephalopathy agent-infected deer, elk, sheep, and cattle. *Journal of* (2002).

262. USGS. Chronic Wasting Disease. *http://www.nwhc.usgs.gov/disease_information/chronic_wasting_disease/* (2013).

263. Sohn, H., Kim, J., Choi, K., Nah, J. & Joo, Y. A case of chronic wasting disease in an elk imported to Korea from Canada. *The Journal of* (2002).

264. Anderson, W. H. Early perceptions of an epidemic. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363,** 3675-3678 (2008).

265. Gajdusek, C. *Discussion on kuru, scrapie and the experimental kuru-like syndrome in chimpanzees*. *1967* (1967).

266. Klitzman, R. L., Alpers, M. P. & Gajdusek, D. C. The Natural Incubation Period of Kuru and the Episodes of Transmission in Three Clusters of Patients. *Neuroepidemiology* **3,** 3-20 (1984).

267. Collinge, J. *et al.* A clinical study of kuru patients with long incubation periods at the end of the epidemic in Papua New Guinea. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363,** 3725-3739 (2008).

268. Whitfield, J. *et al.* Kuru in the 21st century—an acquired human prion disease with very long incubation periods. *The Lancet* **367,** 2068-2074 (2006).

269. Lee, H. & Cervenáková, L. Increased susceptibility to Kuru of carriers of the PRNP 129 methionine/methionine genotype. *Journal of Infectious* (2001).

270. Collinge, J., Powell, J. & Anderton, B. *Prion diseases of humans and animals. 1992* (1992).

271. Gajdusek, D. C. & Zigas, V. Kuru. *The American journal of medicine* **26,** 442-469 (1959).

272. LECOURS, A. & GAJDUSEK, D. Further Observations on the Pathology of Kuru (A Study of the Two Ceredka in Serial Section). *of Neuropathology &* (1967).

273. Beck, E. *et al.* The pathogenesis of transmissible spongiform encephalopathy an ultrastructural study. *Brain* (1982).

274. Dimitz, L. Bericht des Vereines fur Psychiatrie und Neurologie in Wien (Vereinsjahr 1912/13). *Jahrb. Psychiatr. Neurol* **11,** 384

275. Gerstmann, J., Sträussler, E. & Scheinker, I. Über eine eigenartige hereditär-familiäre Erkrankung des Zentralnervensystems. *Zeitschrift für die gesamte* (1935).

276. Lugaresi, E. *et al.* Fatal Familial Insomnia and Dysautonomia with Selective Degeneration of Thalamic Nuclei. *New England Journal of Medicine* **315,** 997-1003 (1986).

277. Kirschbaum, W. Zwei eigenartige Erkrankungen des Zentralnervensystems nach Art der spastischen Pseudosklerose (Jakob). *Zeitschrift für die gesamte Neurologie und Psychiatrie* (1924).

278. 1 An Introduction to Prion Biology and Diseases. *Cold Spring Harbor Monograph Archive* (2004).

279. Collinge, J., Owen, F., Lofthouse, R., Shah, T. & Harding, A. Diagnosis of Gerstmann-Sträussler syndrome in familial dementia with prion protein gene analysis. *The Lancet* (1989).

280. Montagna, P., Cortelli, P., Avoni, P. & Tinuper, P. Clinical features of fatal familial insomnia: phenotypic variability in relation to a polymorphism at codon 129 of the prion protein gene. *Brain* (1998).

281. Hsiao, K., Meiner, Z., Kahana, E. & Cass, C. Mutation of the prion protein in Libyan Jews with Creutzfeldt–Jakob disease. *England Journal of* 1-7 (1991).

282. Brown, P., Goldfarb, L. & Gibbs, C., Jr. The phenotypic expression of different mutations in transmissible familial Creutzfeldt-Jakob disease. *European Journal of* (1991).

283. Mastrianni, J., Curtis, M. & Oberholtzer, J. Prion disease (PrP-A117V) presenting with ataxia instead of dementia. *Neurology* (1995).

284. Piccardo, P., Dlouhy, S. & Lievens, P. Phenotypic variability of Gerstmann-Straussler-Scheinker disease is associated with prion protein heterogeneity. *of Neuropathology &* (1998).

285. Hsiao, K., Cass, C., Schellenberg, G. & Bird, T. A prion protein variant in a family with the telencephalic form of Gerstmann-Sträussler-Scheinker syndrome. *Neurology* (1991).

286. Barbanti, P., Fabbrini, G., Salvatore, M. & Petraroli, R. Polymorphism at codon 129 or codon 219 of PRNP and clinical heterogeneity in a previously unreported family with Gerstmann-Straussler-Scheinker disease (PrP- …. *Neurology* (1996).

287. Kong, Q., Surewicz, W. & Petersen, R. B. 14 Inherited Prion Diseases. *Cold Spring Harbor* (2004).

288. Si, K., Choi, Y., White-Grindley, E. & Majumdar, A. Aplysia CPEB Can Form Prion-like Multimers in Sensory Neurons that Contribute to Long-Term Facilitation. *Cell* (2010).

289. Gambetti, P., Parchi, P., Petersen, R. B. & Chen, S. Fatal Familial Insomnia and Familial Creutzfeldt-Jakob Disease: Clinical, Pathological and Molecular Features. *Brain* (1995).

290. Mastrianni, J. The genetics of prion diseases. *Genetics in Medicine* (2010).

291. Gambetti, P. *et al.* 13 Inherited Prion Diseases. *Cold Spring Harbor Monograph Archive* **38,** 509-583 (1999).

292. Lloyd, S., Mead, S. & Collinge, J. Genetics of prion disease. *Prion Proteins* 1-308 (2011).

293. Brown, P., Goldfarb, L., Kovanen, J. & Haltia, M. Phenotypic characteristics of familial Creutzfeldt-Jakob disease assoicated with the codon 178AsnPRNP mutation. *Annals of* (1992).

294. Brown, P., Cathala, F., Raubertas, R. & Gajdusek, D. The epidemiology of Creutzfeldt-Jakob disease Conclusion of a 15-year investigation in France and review of the world literature. *Neurology* (1987).

295. Will, R. G., Alperovitch, A., Poser, S. & Pocchiari, M. Descriptive epidemiology of Creutzfeldt-Jakob disease in six european countries, 1993–1995. *Annals of* (1998).

296. Will, R. G., Matthews, W. B., Smith, P. G. & Hudson, C. A retrospective study of Creutzfeldt-Jakob disease in England and Wales 1970-1979. II: Epidemiology. *Journal of Neurology, Neurosurgery & Psychiatry* **49,** 749-755 (1986).

297. Zerr, I. & Poser, S. Clinical diagnosis and differential diagnosis of CJD and vCJD. *APMIS* **110,** 88-98 (2002).

298. Will, R. G., Alpers, M. & Dormont, D. 12 Infectious and Sporadic Prion Diseases. *Cold Spring Harbor* (1999).

299. Brown, P., Cathala, F. & Castaigne, P. Creutzfeldt-Jakob disease: Clinical analysis of a consecutive series of 230 neuropathologically verified cases. *Annals of* (1986).

300. Lasmézas, C. I. *et al.* BSE transmission to macaques. *Nature* **381,** 743-4 (1996).

301. Jones, D. & Nevin, S. Rapidly progressive cerebral degeneration (subacute vascular encephalopathy) with mental disorder, focal disturbances, and myoclonic epilepsy. *Journal of Neurology* (1954).

302. Duffy, P. *et al.* Letter: Possible person-to-person transmission of Creutzfeldt-Jakob disease. *The New England journal of medicine* **290,** 692-3 (1974).

303. Brown, P., Preece, M. & Will, R. G. ‘ Friendly fire’ in medicine: Hormones, homografts, and Creutzfeldt-Jakob disease. *The Lancet* (1992).

304. Martinez-Lage, J., Poza, M. & Sola, J. Accidental transmission of Creutzfeldt-Jakob disease by dural cadaveric grafts. *Journal of Neurology* (1994).

305. Bernoulli, C. *et al.* Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery. *Lancet* **1,** 478-9 (1977).

306. Houston, F., McCutcheon, S. & Goldmann, W. Prion diseases are efficiently transmitted by blood transfusion in sheep. (2008).

307. Foster, J. & Chong, A. Transmission of prion diseases by blood transfusion. *Journal of General* (2002).

308. Winter, M. Eight New Hampshire patients and five elsewhere are notified of possible Creutzfeldt-Jakob infection. *http://www.usatoday.com/story/news/nation/2013/09/04/new-hampshire-hospital-fatal-brain-disease/2764645/* (2013).

309. Castilla, J., Saá, P., Hetz, C. & Soto, C. In Vitro Generation of Infectious Scrapie Prions. *Cell* **121,** 195-206 (2005).

310. Deleault, N., Lee, K. S., Harris, B., Caughey, B. & Rees, J. A simplified recipe for prions. in *PNAS* **104,** 9551-9552 (2007).

311. Wang, F., Wang, X., Yuan, C. & Ma, J. Generating a prion with bacterially expressed recombinant prion protein. *Science* (2010).

312. Edgeworth, J. A. *et al.* Spontaneous generation of mammalian prions. in *PNAS* **107,** 14402-14406 (National Acad Sciences, 2010).

313. Zhang, Z. *et al.* De novo generation of infectious prions with bacterially expressed recombinant prion protein. *The FASEB journal* **27,** 4768-4775 (2013).

314. Sigurdson, C. J. *et al.* De novo generation of a transmissible spongiform encephalopathy by mouse transgenesis. in *PNAS* **106,** 304-309 (National Acad Sciences, 2009).

315. Westaway, D., DeArmond, S. & Cayetano-Canlas, J. Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild-type prion proteins. *Cell* (1994).

316. Chiesa, R., Piccardo, P., Ghetti, B. & Da Harris. Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. *Neuron* (1998).

317. Collinge, J. & Clarke, A. R. A general model of prion strains and their pathogenicity. *Science* 1-8 (2007).

318. Hsiao, K. *et al.* Spontaneous neurodegeneration in transgenic mice with mutant prion protein. *Science* **250,** 1587-1590 (1990).

319. Chiesa, R., Drisaldi, B. & Quaglio, E. Accumulation of protease-resistant prion protein (PrP) and apoptosis of cerebellar granule cells in transgenic mice expressing a PrP insertional mutation. in *Proceedings of the* (2000).

320. Soto, C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nature Reviews Neuroscience* (2003).

321. Chiti, F. & Dobson, C. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem* (2006).

322. Blake, C., Serpell, L. & Sunde, M. A molecular model of the amyloid fibril. *Of Amyloid Fibrils* (1996).

323. Soto, C., Estrada, L. & Castilla, J. Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. *Trends in biochemical sciences* **31,** 150-155 (2006).

324. Baker, H., Ridley, R., Duchen, L. & Crow, T. Induction of β (a4)-amyloid in primates by injection of Alzheimer's disease brain homogenate. *Molecular* (1994).

325. Kane, M. D. *et al.* Evidence for Seeding of β-Amyloid by Intracerebral Infusion of Alzheimer Brain Extracts in β-Amyloid Precursor Protein-Transgenic Mice. *The Journal of neuroscience* **20,** 3606-3611 (2000).

326. Meyer-Luehmann, M., Coomaraswamy, J. & Bolmont, T. Exogenous induction of cerebral ß-amyloidogenesis is governed by agent and host. *Science* 1781-1784 (2006).

327. Laurén, J., Da Gimbel, Nygaard, H. & Gilbert, J. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-&bgr; oligomers. *Nature* (2009).

328. Da Gimbel, Nygaard, H. & Coffey, E. Memory impairment in transgenic Alzheimer mice requires cellular prion protein. *The Journal of* (2010).

329. You, H., Tsutsui, S. & Hameed, S. Aβ neurotoxicity depends on interactions between copper ions, prion protein, and N-methyl-D-aspartate receptors. in *Proceedings of the* (2012).

CHAPTER 2:

IN VITRO STRAIN ADAPTATION OF CWD PRIONS BY SERIAL PROTEIN MISFOLDING CYCLIC AMPLIFICATION*

SUMMARY

We used serial protein misfolding cyclic amplification (sPMCA) to amplify the D10 strain of CWD prions in a linear relationship over two logs of D10 dilutions. The resultant PMCA-amplified D10 induced terminal TSE disease in CWD-susceptible Tg(cerPrP)1536 mice with a survival time approximately 80 days shorter than the original D10 inoculum, similar to that produced by in vivo sub-passage of D10 in Tg(cerPrP)1536 mice. Both *in vitro*-amplified and mouse-passaged D10 produced brain lesion profiles, glycoform ratios and conformational stabilities significantly different than those produced by the original D10 inoculum in Tg(cerPrP)1536 mice. These findings demonstrate that sPMCA can amplify and adapt prion strains *in vitro* as effectively as and much more quickly than *in vivo* strain adaptation by mouse passage. Thus sPMCA may represent a powerful tool to assess prion strain adaptation and species barriers *in vitro*.

INTRODUCTION

According to the prion hypothesis, a proteinacious pathogen devoid of instructional nucleic acid initiates and propagates transmissible spongiform encephalopathies (TSEs), a group of invariably fatal, infectious neurological diseases [1] characterized by auto-conversion of the normal host cellular prion protein (PrP^C) into a

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misfolded, insoluble, proteinase K (PK)-resistant form (PrP^{RES}). Mounting biochemical and biological evidence supports the prion hypothesis[1-4]. Prion infectivity correlates with PrP^{RES} in brain homogenates from animals afflicted with TSEs, including sheep scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD, [5-10]. More recently, synthetic prions made from truncated recombinant PrP have been shown to be infectious when inoculated into transgenic mice expressing the same isoform and in wild type mice upon subsequent passage [11]. Additional evidence has come from several reports that demonstrate *in vitro* generation of infectious hamster prions using serial protein misfolding cyclic amplification (sPMCA), a highly efficient amplification method employing repeated cycles of incubation of prions with normal brain homogenate (NBH) as a source of PrP^{C} substrate to grow existing prion templates, and sonication to break the resulting large aggregates into smaller, more numerous prion templates [12-15]. Serial PMCA of PrP^{RES} from mouse-adapted scrapie (PrP^{Sc}) and CWD prions (PrP^{CWD}) has recently been described[16,17] although infectivity of these amplified materials was not assessed.

The unique etiology of mammalian prion diseases complicates characterization, identification, and even the definition of prion strains. Traditionally prion strains have been typed according to host range, incubation time to terminal disease and neuroanatomical lesion profiles based on seminal work comparing human and animal TSEs [18-21]. Other criteria have been developed based on biochemical and biophysical properties of prions to investigate heritable structural differences among different prion strains, including size and extent of the PK-resistant core [22-24], glycoform ratio and conformational stability upon chemical or thermal denaturation [25-31]. These parameters

have proven useful for identification of prion strains with similar origins, host ranges and pathology and to predict transmission barriers to heterologous host species [32-34].

Experimental inoculation of animals represents the most reliable and accepted measure of efficiency of strain adaptation and transmission of prions into new hosts [7,35-39]. However, these experiments often require extraordinarily long incubation periods, even by prion experimental standards, to fully assess strain adaptation and species barriers. Here we report efficient linear amplification of PrP^{CWD} by sPMCA resulting in *in vitro* generation of infectious CWD prions. Remarkably, we observed a drastic, nearly identical reduction in incubation time to terminal disease of CWD-susceptible Tg(cerPrP)1536 mice inoculated with *in vitro*-amplified or mouse-passaged prions from the D10 isolate of CWD prions when compared to the original D10 inoculum. *In vitro*-amplified and *in vivo*-passaged D10 also shared similar neuropathological and biochemical characteristics that were significantly different than the original D10 strain, more closely resembling the RML strain of mouse-adapted scrapie prions. By all accepted parameters used to characterize prion strains that we examined, sPMCA adapted the D10 CWD strain as efficiently as passage in Tg(cerPrP)1536 mice, and represents a powerful, efficient tool for assessing strain adaptation and species barriers *in vitro*.

MATERIALS AND METHODS

Mice

FVB mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Tg(cerPrP)1536 and Tga20 mice were generated as previously described [10,40]. All mice were bred and maintained at Lab Animal Resources, accredited by the Association for

Assessment and Accreditation of Lab Animal Care International, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University.

Sources and preparation of prion inocula

The D10 isolate of CWD prions (D10) and the Rocky Mountain Lab strain of mouse-adapted scrapie prions passage 5 (RML) were previously described[10,41]. D10 was then propagated through Tg(cerPrP)1536 mice or amplified *in vitro* by serial protein misfolding cyclic amplification (sPMCA). 10% brain homogenates were prepared in PBS or PMCA buffer (4mM EDTA, 150 mM NaCl in PBS). Densitometric analysis of PK-digested samples on western blots revealed similar band intensities of equal dilutions of D10, mouse-passaged D10, amplified 10^{-24} D10 and RML. We therefore diluted equal volumes of each inoculum 1:10 in 320 mM sucrose supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (Gibco) in PBS thirty minutes prior to intracerebral inoculations.

Preparation of normal brain homogenate (NBH) and PMCA

Mice were euthanized and perfused with 30 ml 5mM EDTA in PBS. Whole brains were removed and immediately frozen in liquid nitrogen. Brains were weighed and transferred into 1.5 mL Eppendorf tubes with 2.5 mm glass beads. PMCA buffer with 2X Complete Protease Inhibitor Cocktail (Roche) was added to make a 20 % w/v solution. Samples were homogenized for 20 s at 4.5 m/s in a FastPrep machine (Biogene), cooled on ice for two minutes and centrifuged at 14,000 x g for 10 s to reduce foaming. This process was repeated twice. NBH was diluted to a 10% w/v solution by adding an equal volume of PMCA conversion buffer containing 2% Triton X-100 and incubated on

ice for 20 min. NBH was clarified by centrifuging for 30 s at 1,500 x g and supernatants were aliquoted to into new tubes and stored at -70^{o}C. PMCA was modified from a previous protocol[17]. Briefly, 50 µL samples of D10 diluted into 10% NBH were placed into wells of a 96-well microplate. The entire plate was suspended in the water bath of a 3000MP sonicator (Misonix) and sonicated at 70- 85% maximum power for 40 seconds, followed by a 30-minute incubation at 37^{O}C. This cycle was repeated 96 times. For sPMCA, 50 µL of a 10^{-3} D10 dilution in NBH was subjected to PMCA. 50 µL of a 10^{-3} dilution of amplified material in fresh NBH was subjected to another PMCA round. This process was repeated for a total of eight rounds.

PK digestion and western blotting

Samples were digested with 50 µg/ml PK (Roche) for 30 min at 37^{o}C. The reaction was stopped by adding lithium dodecyl sulfate sample loading buffer (Invitrogen) and incubating at 95^{o}C for 5 min. Proteins were electrophoretically separated through 12% sodium dodecyl sulfate-polyacrylamide gels (Invitrogen), and transferred to polyvinylidene difluoride membranes (Millipore). Non-specific membrane binding was blocked by incubation in 5% milk blocking solution (Bio-Rad) for 1 h. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated Bar224 anti-PrP monoclonal antibody (SPI bio) diluted 1:20,000 in Superblock (Pierce), washed 6 x 10 min in PBS with 0.2% Tween 20, and incubated for 5 min with enhanced chemiluminescent substrate (Millipore). Membranes were digitally photographed using the FujiDoc gel documentation system equipped with a cooled charge-coupled diode camera (Fuji). Densitometric analyses were performed using Quantity One software (Bio-Rad).

Prion inoculations and clinical scoring

Mice were anesthetized by Isofluorane inhalation. Thirty microliters of each inoculum was injected intracerebrally 3 mm deep through the coronal suture 3-5 mm lateral of the sagittal suture. Mice were monitored daily for clinical symptoms of prion disease, including tail rigidity, impaired extensor reflex, akinesia, tremors, ataxia, weight loss and paralysis. Mice with any four of these symptoms were scored terminally sick and euthanized.

Histochemistry and immunohistochemistry (IHC)

Tissues were fixed in 10% paraformaldehyde, embedded in paraffin and 5-10 µm sections mounted on glass slides. For PrP staining tissue sections were deparaffinized, treated with concentrated formic acid for thirty minutes, then autoclaved at 121^{o}C in target retrieval solution (Dako) for 2 h, washed 2 x 2 min in 1X PBS, treated with 0.3% H_20_2 in methanol for 10 min and blocked for 15 min with 1% BSA in PBS. Sections were then incubated with horseradish peroxidase-conjugated anti-PrP Bar 224 monoclonal antibody diluted 1:100 in antibody diluent (Dako) for 1 hour, rinsed 2 x 2 min in PBS, exposed to a Diaminobenzidine solution (Dako) for 2 min, rinsed 2 x 2 min in PBS and counterstained with hematoxylin. Slides were dehydrated in xylene and coverslips mounted with Cytoseal (Richard Allan Scientific). For Thioflavin T staining, sections were treated with 1% Thioflavin T in distilled water for 10 min at room temperature, followed by 1% acetic acid in water for 10 min and then rinsed with distilled water for 5 min. Hematoxylin and Eosin (H&E) and glial fibrillary acidic protein (GFAP) staining was performed on a NexES automated IHC stainer (Ventana Medical systems, Inc. Tucson, AZ). Sections were stained with H&E for 4 min at room temperature. Sections were

stained with rabbit polyclonal antisera against GFAP (diluted 1: 8) for 10 min at 37^{o}C followed by Biotinylated goat anti-rabbit Ig (mouse/rat adsorbed) for 8 min, then counterstained with hematoxylin for 4 min. Sections were visualized and digitally photographed using an Olympus BX60 microscope equipped with a cooled charge-coupled diode camera.

Lesion profiling

Brain lesion profiling was performed as previously described [42] with slight modifications. Ten neuroanatomic regions were identified in medial sagittal brain sections from at least six mice of each group: 1-dorsal medulla, 2-cerebellum, 3-superior colliculus, 4- red nucleus, 5-hypothalamus, 6-hippocampus, 7-thalamus, 8-cerebral cortex, 9-primary somatosensory cortex and 10-caudate-putamen. Four investigators blinded to group identification scored each region for vacuolation, astrogliosis and PrP^{RES} deposition using the following severity scale: normal (0-1), mild (2), moderate (3) and severe (4). The average of the sum of the three scores constitutes the severity score for each region.

Conformational stability assay

Conformational stability assays were modified from a previous protocol[43]. 15 μl aliquots of brain homogenates were incubated for 1 h at room temperature with increasing concentrations of guanidine hydrochloride (GdnHCl, Sigma) ranging from 0 to 3.75 M in 0.375 M increments. Samples were precipitated in ice-cold methanol at -20^{o}C overnight, centrifuged at 13,000 X g for 30 min at 4 ^{o}C. Pellets were washed in PMCA buffer and centrifuged 3x, resuspended in 18 μl of PMCA buffer, PK-digested and western blotted. Conformational stability was quantified by densitometric analyses

of western blots, plotting the mean percentage of PrP^{RES} remaining ± SD as a function of GdnHCl concentration, and using fourth order polynomial equations and nonlinear regression (GraphPad Prism) to fit denaturation curves for each prion strain.

Statistical analyses

One-way ANOVA with Tukey post-test analysis was performed using GraphPad Prism.

RESULTS

Linear amplification of PrP^{CWD}

In vitro amplification of PK-resistant PrP (PrP^{RES}) from CWD prions using protein misfolding cyclic amplification (PMCA) has recently been reported[17]. We extend this work in several ways. We first tested whether NBH expressing heterologous PrP^{C} could be used to amplify PrP^{RES}. We performed PMCA of D10 and RML serially diluted into NBH from wild type FVB mice (Figure 1A) and TgA20 mice expressing 4-5-fold more mouse PrP^{C} ($moPrP^{C}$, Figure 1B). Both $moPrP^{C}$ substrates supported amplification of scrapie PrP^{RES} (PrP^{Sc}), but not CWD PrP^{RES} (PrP^{CWD}). We then performed PMCA on D10 and RML samples serially diluted into Tg(cerPrP)1536 NBH expressing five-fold more cervid PrP^{C} (cerPrP) than FVB mice express $moPrP^{C}$ (C). After PMCA we detected a 6×10^{6}-fold dilution of PrP^{CWD} (lane 8), whose band intensity matched that of the 9×10^{2}-fold dilution of unamplified D10 (lane 3), thus yielding an approximately 6666-fold increase in PrP^{CWD}. Tg(cerPrP)1536 NBH failed to support PrP^{Sc} amplification. Quantitative analyses of band intensities from three experiments demonstrated a consistent, rapid decrease in band intensity of unamplified PrP^{CWD} dilutions, with complete loss of signal at 10^{-3} dilutions (D). Plotting -fold amplification as

a function of –fold dilution of D10 on a log-log scale reveals a linear relationship between 10^3 and 2 x 10^5-fold dilution of D10 (E).

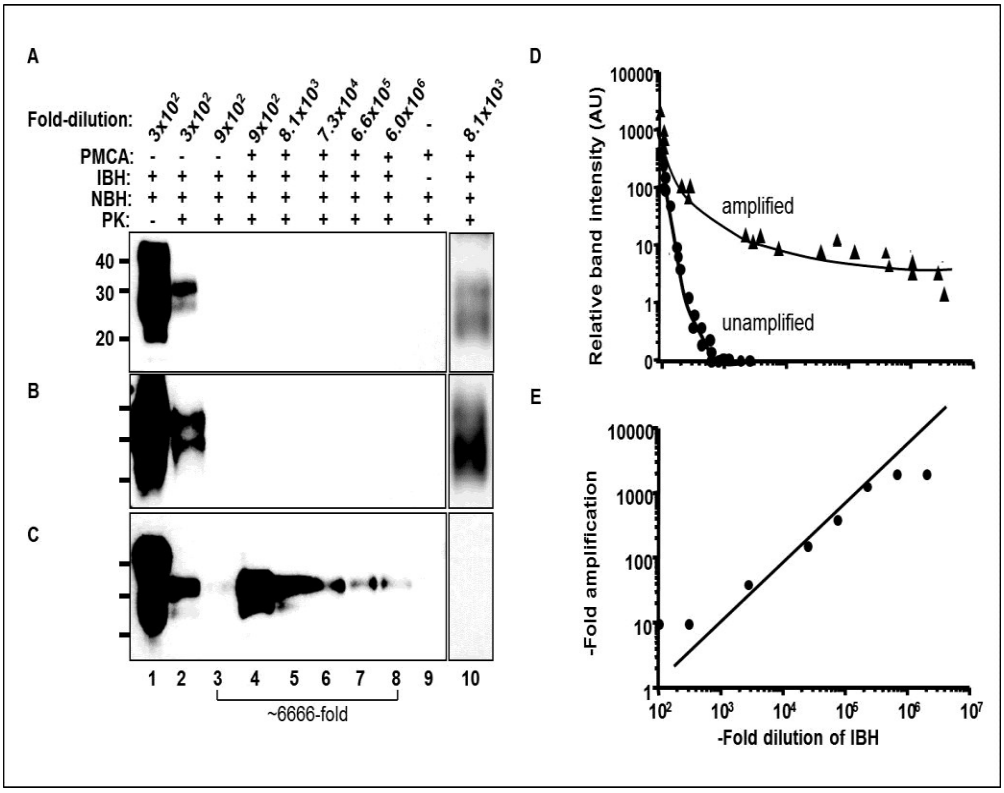

Figure 2.1. Protein misfolding cyclic amplification of chronic wasting disease prions. Three-fold serial dilutions of infected brain homogenate from the D10 isolate of CWD prions (D10, lanes 1-9) or Rocky Mountain lab strain of mouse-adapted scrapie prions (RML, lane 10) diluted 1:3 x 10^2 into normal brain homogenate (NBH) were either snap-frozen (lanes 1-3) or subjected to 96 cycles of PMCA (lanes 4-10). Proteinase K (PK) digestion and western blotting of samples reveal that wild type NBH (A) or TgA20 NBH (B) supported PrP^{Sc} (lane 10), but not PrP^{CWD} (lanes 4-9) amplification, whereas Tg(cerPrP)1536 NBH (C) efficiently amplified PrP^{CWD} (lanes 4-9), but not PrP^{Sc} (lane 10), resulting in approximately 6666-fold PrP^{CWD} amplification after one PMCA round (compare lane 3 to 8). Molecular weight markers are shown in kDa to the left of the blots. (D) Quantification of band intensities demonstrates a dramatic decrease in signal intensity upon dilution of D10 without PMCA, and sustained intensity to 6 x 10^6 -fold dilution with PMCA. (E) Plotting –fold amplification as a function of D10 dilution reveals a linear relationship over two logs of D10 dilutions. Data are representative of at least three independent experiments.

sPMCA or mouse passage of D10 shortens mean incubation time to terminal prion disease

To determine whether amplified PrP^{CWD} is infectious, we used serial PMCA (sPMCA) to generate PrP^{CWD} *in vitro*. From our quantification data, repeated 10^{-3} dilution and amplification should maintain sPMCA in the linear range of PrP^{CWD} amplification (Figure 1E). Beginning with a 10^{-3} D10 dilution, we achieved a ten-fold amplification efficiency after one PMCA round, producing PrP^{CWD} equivalent to a 10^{-2} dilution. Re-diluting this material 10^{3}-fold produced a 10^{-5} dilution equivalent. We then amplified this material approximately 10^{3}-fold, again producing a 10^{-2} equivalent dilution. We continued this sPMCA strategy for six more rounds, oscillating between 10^{-5} and 10^{-2} equivalent dilutions upon re-dilution and PMCA to arrive at an overall D10 dilution of 10^{-24}, a point at which the original PrP^{RES} inoculum has been estimated to be lost, and only amplified PrP^{RES} is present in the sample [12]. Western blot analysis demonstrated the maintenance of a strong PrP^{RES} signal in the amplified product throughout sPMCA, whereas unamplified samples were undetectable (figure 2A). Densitometric analysis revealed similar band intensities for PK-digested D10, amplified 10^{-24} D10 and mouse-passaged D10, with RML producing approximately half the intensity of the other three inocula (Figure 2B). We therefore inoculated Tg(cerPrP)1536 mice intracerebrally with 30 µl of each D10-derived inoculum diluted 1:10 in sterile 320 mM sucrose supplemented with antibiotics in PBS. All mice inoculated with amplified D10 contracted neurologic disease consistent with TSE with a mean incubation time to terminal illness of 169 ± 4 days post inoculation (DPI), while all mice inoculated with unamplified D10 are currently asymptomatic at >500 DPI (Table 1). Surprisingly, Tg(cerPrP)1536 mice

inoculated with the original D10 strain contracted disease 82 days later (251 ± 3 DPI) than amplified D10-inoculated mice. Inoculation of Tg(cerPrP)1536 mice with mouse-passaged D10 (D10 Passage 1) resulted in an 80-day shortening of incubation time (171 ± 3 DPI) that closely matched the incubation time for amplified D10-inoculated mice. Wild type mice inoculated with D10 and Tg(cerPrP)1536 mice inoculated with RML are currently asymptomatic at >500 and 300 DPI, respectively. Wild type mice inoculated with 30 µL of RML contracted scrapie 156 ± 8 DPI, which is very similar to incubation times observed for mice inoculated with *in vitro*-amplified and mouse-adapted D10. Taken together, these data comparing primary passage of amplified D10, primary passage of D10 and secondary D10 passage using mean incubation time to terminal disease, the accepted benchmark for prion strain characterization, strongly suggest that strain adaptation of D10 CWD prions occurred *in vitro* and *in vivo*. However, because PrP^{RES} concentration does not always directly correlate with prion infectivity [44,45], normalizing prion inoculum by estimating PrP^{RES} content may have resulted in imprecise measurement of prion doses that could explain the differences in mean incubation times that we observed in the mouse bioassay. We therefore analyzed these inocula by neuropathological and biochemical criteria previously used to assess other prion strain properties.

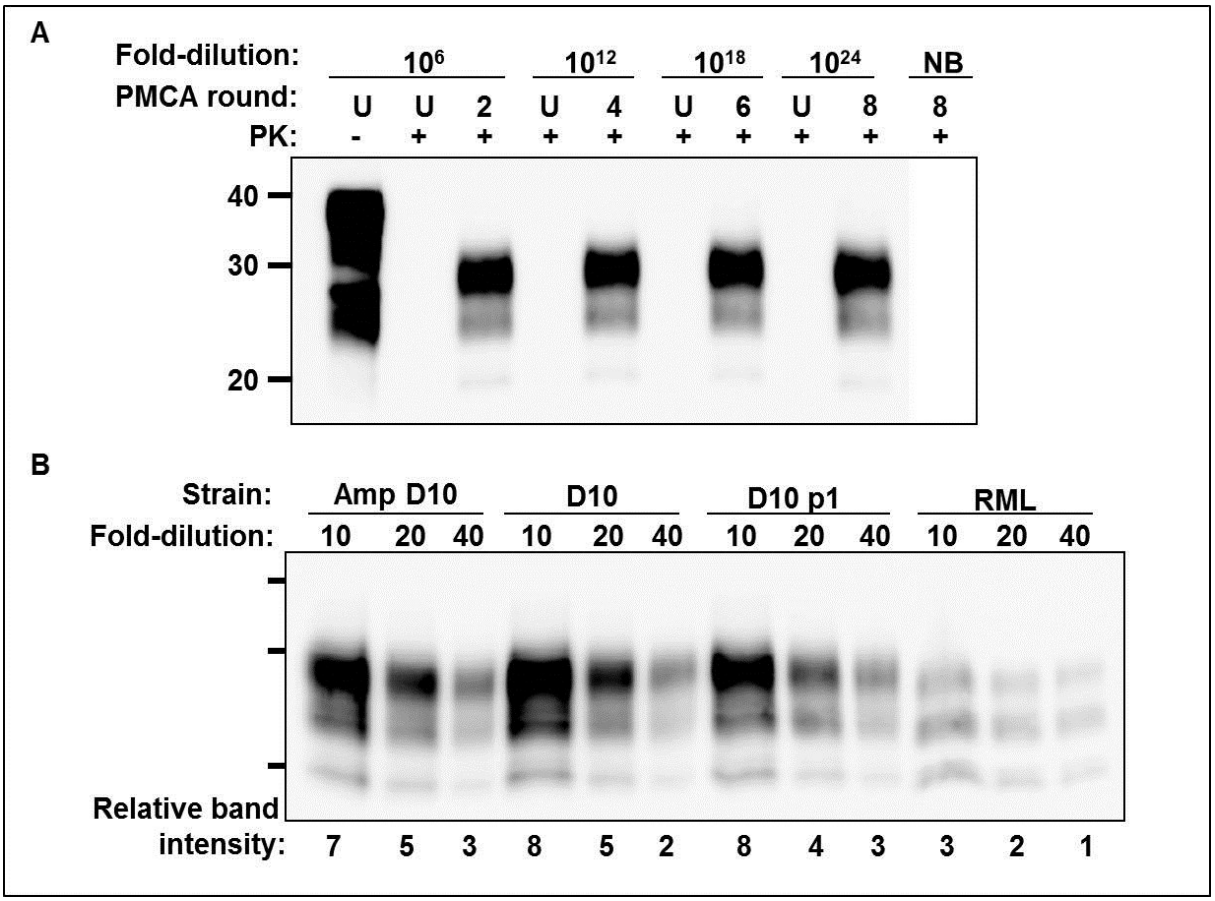

Figure 2.2. (A) Serial PMCA sustains CWD prion amplification. A 10^3-fold dilution of D10 was subjected to serial PMCA (sPMCA). After each round, the sample was re-diluted 1:10^3 in fresh NBH and subjected to an additional 96 PMCA cycles. Fold-dilutions refer to the overall dilution relative to the starting dilution and were detected at the indicated round. Unamplified samples at each dilution (U) and unspiked normal brain (NB) were not detected. (B) Western blot signal intensity comparison of 10, 20 and 40-fold dilutions of amplified D10, original D10, mouse-passaged D10 and RML inocula.

Table 2.1. Strain adaptation of CWD Prions.

	Tg1536		Wild Type	
Inoculum[a]	Incidence[b]	DPI ± SD[c]	Incidence	DPI ± SD
Amplified 10^{-24} D10	6/6	169 ± 4[d]	ND[e]	ND
Unamplified 10^{-24} D10	0/8	>500	ND	ND
D10	8/8	251 ± 3	0/5	>500
D10 Passage 1	6/6	171 ± 3[d]	ND	ND
RML 5.0	0/6	>300	10/10	156 ± 8

[a]Mice were inoculated intracerebrally
[b]Incidence = number of terminally sick mice/ number of animals inoculated
[c]DPI ± SD, days post inoculation to terminal disease ± standard deviation
[d]$p < 0.01$ compared to Tg1536 inoculated with D10
[e]ND, no data

sPMCA and mouse passage of D10 changes CWD neuropathology

To compare CWD neuropathology of terminally sick mice inoculated with *in vitro*-amplified and mouse-passaged D10 to mice inoculated with the original D10 strain, we analyzed brain sections for vacuolation, astrogliosis, PrP^{RES} deposition and amyloidogenicity (Figure 3). Brains of Tg(cerPrP)1536 mice inoculated with amplified D10 (Amp D10 p1 mice) exhibited little to no neuronal cell loss (Figure 3A), mild vacuolation (figure 3A-C), disseminated astrogliosis (figure 3B) and diffuse PrP^{CWD} deposits (figure 3C) in the hippocampal region (Figure 3A-C). These deposits stained poorly with Thioflavin T (Figure 3D), indicating little or no amyloid structure. We observed increased neuropathology in the cerebellum, with more vacuolation (figure 3E-G), astrogliosis (figure 3F) and PrP^{RES} deposition (figure 3G). Thioflavin T stained a few deposits, revealing some amyloid structure (Figure 3H). In contrast, hippocampal sections of Tg(cerPrP)1536 mice inoculated with original D10 inoculum (D10 p1 mice)

displayed extremely severe vacuolation (Figure 3I-K), neuronal cell loss (Figure 3I) and astrogliosis (Figure 3J). Numerous PrP^{CWD} deposits appeared more focal and dense (Figure 3K) and Thioflavin T stained these punctate plaques sharply, revealing a more defined, radially organized amyloid structure (Figure 3L). However, little or no cerebellar neuropathology was evident in these mice compared to Amp D10 p1 mice (figure 3M-P). Upon secondary passage of D10 (D10 p2 mice), we observed decreased neuropathology in the hippocampus similar to that found in Amp D10 p1 mice, with little or no vacuolation or neuronal cell loss (Fig3Q-S). The mild to moderate astrogliosis observed (Figure 3R) was dramatically decreased compared to D10 p1 mice. Little or no PrP^{RES} deposition was evident (Figure 3S) and no Thioflavin T positive staining was observed (Figure 3T). In the cerebellum, we observed an increase in vacuolation, neuronal cell loss (Figure 3U-W) and astrogliosis (Figure 3V). We also observed faintly stained, diffuse PrP^{CWD} deposits (Figure 3W), some of which were stained by Thioflavin T (Figure 3X). These rare amyloid structures appeared more diffuse and lacked the radially structured punctae clearly evident in amyloid from D10 p1 mice (Figure 3L). Hippocampal sections of wild type mice inoculated with RML exhibited vacuolation, neuronal cell loss (Figure 3Y), astrogliosis (Figure 3Z) and diffuse PrP^{Sc} deposition (Figure 3AA) typical of mouse scrapie and reminiscent of neuropathology observed in Amp D10 p1 and D10 p2 brains.

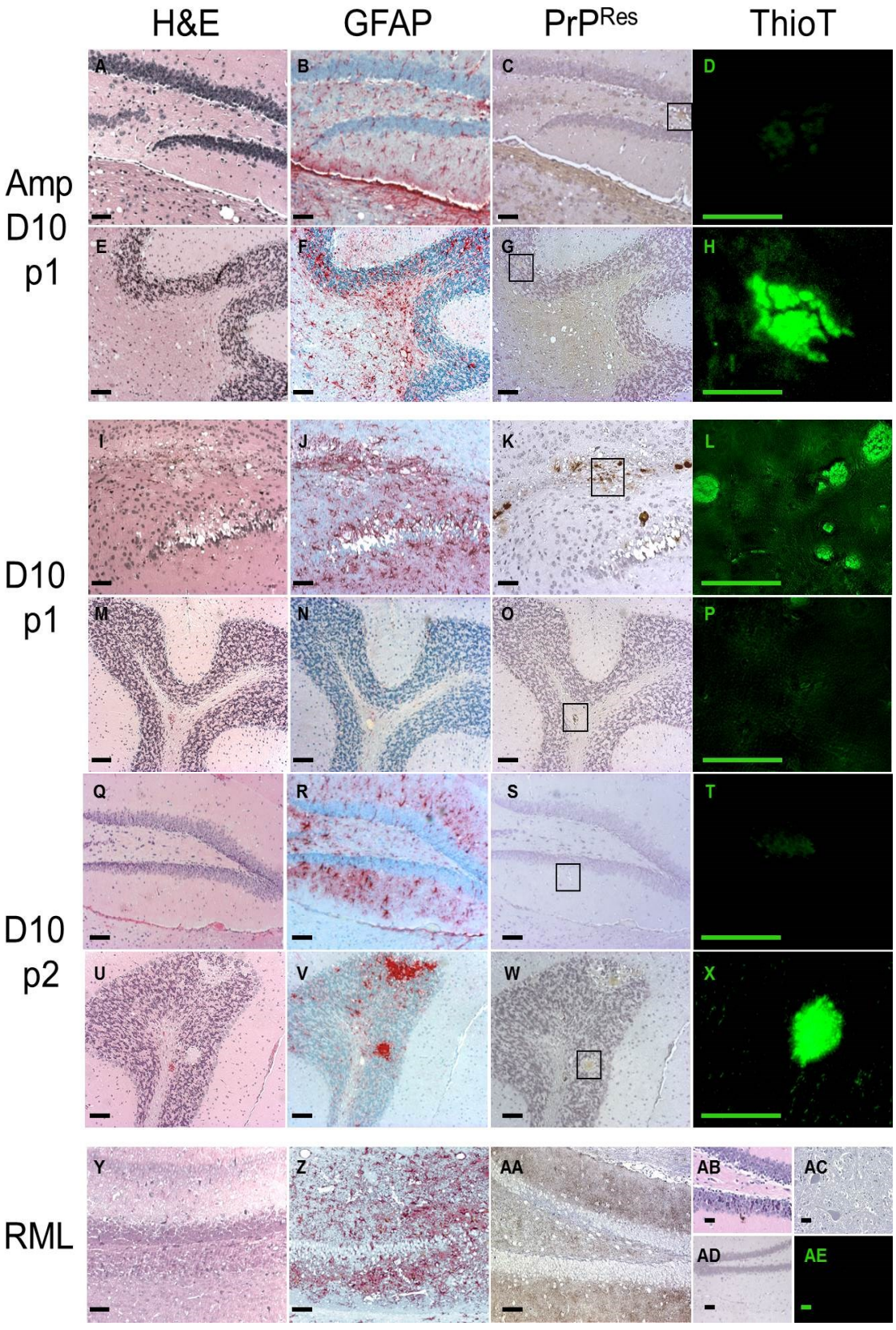
H&E
GFAP
PrPRes
ThioT
Amp D10 p1
D10 p1
D10 p2
RML
A
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Figure 2.3. Strain adaptation changes CWD neuropathology. Brain sections from terminally sick Tg(cerPrP)1536 and wild type mice were assessed for vacuolation (H&E), astrogliosis (GFAP), PrP^{RES} deposition (PrP^{RES}) and amyloidogenicity (ThioT). Boxed areas in the PrP^{RES} panels are shown stained with Thioflavin T to the right. Hippocampal sections from mice inoculated with amplified D10 exhibited little or no neuronal cell loss, mild to moderate vacuolation (A-C), astrogliosis (B), and diffuse PrP^{CWD} deposition in the hippocampus (C) that Thioflavin T failed to stain (D). Cerebellar neuropathology was more pronounced (E-H), with increased vacuolation (E), astrogliosis (F) and PrP^{CWD} deposits (G), some of which were stained with Thioflavin T (H). In contrast, hippocampal sections from mice inoculated with the original D10 isolate exhibited more extensive neuronal cell loss and vacuolation (I-K) and astrogliosis (J), with dense, punctate plaques of PrP^{CWD} (K) that stained sharply with thioflavin T (L), revealing more numerous amyloid structures that appeared punctate and radially organized. Little or no cerebellar neuropathology was observed in the brains of these mice (M-P). Upon second passage (D10 p2), little to no neuronal cell loss and vacuolation (Q-S) and mild to moderate astrogliosis (R) was evident in hippocampal sections, and PrP^{CWD} deposition was much more diffuse (K) and lacked amyloidogenicity (T). However, cerebellar neuropathology was clearly evident, with increased neuronal cell loss, vacuolation (U-W), and PrP^{CWD} deposits (W), some of which were amyloidogenic, but lacked the radially structured, punctate deposits found in D10 p1 plaques. Hippocampal sections from RML-infected wild-type mice exhibited vacuolation (Y) astrogliosis (Z) and diffuse PrP^{Sc} deposition (AA) reminiscent of that produced by amplified D10 p1 and D10 p2 strains of CWD prions. Brain sections from D10-inoculaed $PrP^{-/-}$ mice were stained with H&E (AB), GFAP (AC), anti-PrP (AD) and Thioflavin T (AE) as negative controls. Scale bars, 50 μm.

We examined eight additional brain areas for neuropathology to generate lesion profiles for each inoculation group. We observed increased neuropathology, especially diffuse PrP^{RES} deposition and vacuolation, in the dorsal medulla and cerebellum, with moderate to severe astrogliosis in the superior colliculus, red nucleus and thalamus in amplified and mouse-passaged D10- inoculated Tg(cerPrP)1536 mice and RML-inoculated wild type mice. We observed decreased overall neuropathology in these brain regions, and increased neuropathology in the hippocampus of Tg(cerPrP)1536 mice inoculated with the original D10 strain. Seven lesion profile scores for brains from Amp D10 p1, five for D10 p2 and six for RML infected mice differed from D10 p1 scores without overlapping standard deviations (SDs); while ≤ 3 lesion profile scores differed

without overlapping SDs among brains from Amp D10 p1, D10 p2 and RML infected mice (Figure 4). Overall lesion severity also increased and neuroanatomic location was altered from D10 p1 to D10 p2 and Amp D10 p1 to more closely match severity and location of RML-induced lesions. Taken together, these data indicate that *in vitro* amplification and *in vivo*-passage of D10 changes CWD neuropathology to one that more closely resembles scrapie neuropathology in mice and further support the proposition that D10 strain adaptation has occurred *in vitro* and *in vivo*.

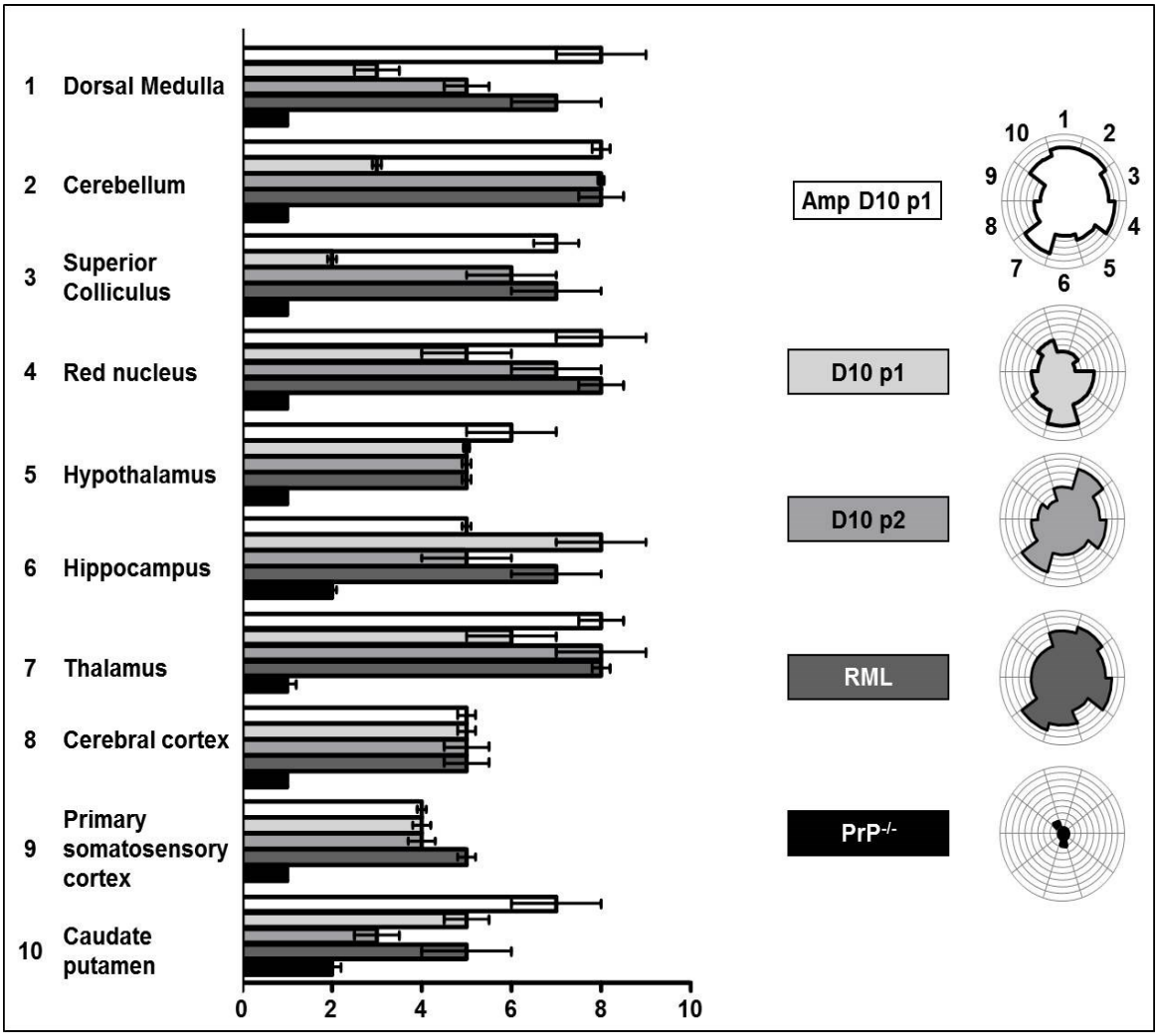


Figure 2.4. Strain adaptation changes CWD lesion profiles. Bar graphs displaying results of lesion profile analyses for each prion strain at the indicated brain regions.

$PrP^{-/-}$ mice inoculated with D10 is shown as a baseline control. Radar plots shown to the right facilitate comparison of overall lesion profiles. Each wedge of the plot represents the indicated brain region and each concentric circle represents a severity point. Amp D10 p1, D10 p2 and RML share similar profiles that are distinct from the D10 p1 profile.

sPMCA and mouse passage of D10 changes PrP^{CWD} glycoform ratios

We next characterized these prion inocula biochemically by assessing PK-resistant fragment sizes and glycoform ratios, which have previously been used to characterize prion strains[26,28]. While analysis of pooled brain homogenate from at least three mice from each group or three PMCA reactions revealed no differences in PK-resistant core fragment sizes, we did detect significant differences in glycoform ratios (Figure 5A). RML consisted of substantial monoglycosylated PrP^{Sc}, while diglycosylated PrP^{CWD} constituted the vast majority of D10. Significant amounts of di- and monoglycosylated PrP^{CWD} was observed in Amp D10 p1. Primary passage of D10 into Tg(cerPrP)1536 mice (D10 p1) shifted the glycoform ratio toward monoglycosylated PrP^{CWD}, which was further increased upon secondary passage (D10 p2). sPMCA of D10 also shifted the glycoform ratio toward monoglycosylated PrP^{CWD}. We did observe some variability in these trends when we analyzed brain homogenates from individual mice, especially among D10 p1 samples (Figure 5B). We therefore quantified band intensities from multiple western blots to more accurately assess glycoform ratios of all prion strains (Figure 5C and D). The RML di: mono: unglycosylated PrP^{Sc} ratio of 0.24 ± 0.09: 0.53 ± 0.12: 0.23 ± 0.08 was drastically different from those of all other strains ($p<0.01$). D10 consisted of a PrP^{CWD} glycoform ratio of 0.71 ± 0.05: 0.26 ± 0.05: 0.03 ± 0.01. sPMCA of D10 produced amplified material with a glycoform ration of 0.74 ±0.04: 0.23 ±0.03: 0.03 ±0.01, nearly identical to D10. Passage of serially amplified D10 into Tg(cerPrP)1536 mice (Amp D10 p1) significantly changed the glycoform ratio to 0.49 ±

0.05: 0.35 ± 0.04: 0.16 ± 0.03 ($p<0.01$ compared to D10), decreasing overall glycosylation. A similar phenomenon occurred upon *in vivo* D10 adaptation. Primary passage into Tg(cerPrP)1536 mice (D10 p1) slightly altered the glycoform ratio to 0.66 ± 0.21: 0.24 ± 0.14: 0.10 ± 0.08, while secondary passage (D10 p2) significantly shifted the ratio to 0.57 ± 0.07: 0.33 ± 0.06: 0.10 ± 0.07 ($p<0.05$ for di- and monoglycosylated PrP^{CWD} compared to D10). These data reveal similar glycoforms ratios of Amp D10 p1 and D10 p2 prion inocula that are significantly different from D10 and D10 p1 inocula.

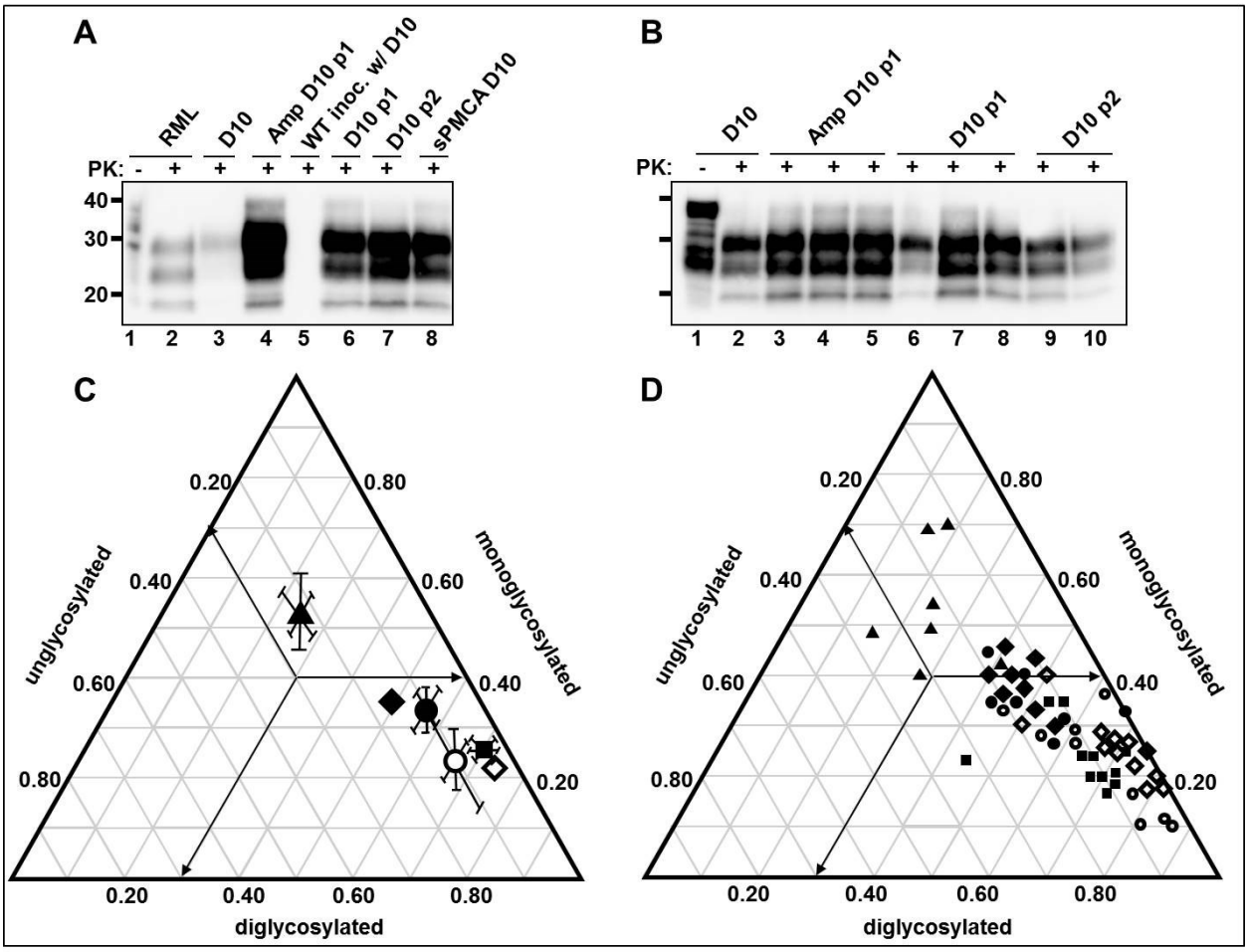


Figure 2.5. Strain adaptation changes PrP^{CWD} glycoform ratios. (A) Western blot of pooled brain homogenates (BH) from at least three mice demonstrates that the RML strain of mouse-adapted scrapie consists of substantial monoglycosylated PrP^{Sc} (lane 2). The original D10 isolate of CWD prions contains primarily diglycosylated PrP^{CWD} (lane 3). Pooled BH from terminally sick mice inoculated with *in vitro* amplified D10

contains increased monoglycosylated PrP^{CWD} compared to D10 (lane 4). Inoculation of D10 into Tg(cerPrP)1536 mice shifts glycoform ratios toward the monoglycosylated form upon primary (lane 6) and especially secondary (lane 7) passage. Similarly, sPMCA of D10 shifts the ratio toward the monoglyosylated form (lane 8). Pooled BH from asymptomatic wild type mice inoculated with D10 contained no detectable PrP^{CWD} (lane 4). (B) Western blot of individual BH confirms increased monoglycosylated PrP^{CWD} of Amp D10 p1 (lanes 3-5) and D10 p2 (lanes 9 and 10), while D10 p1 is more variable (lanes 6-8). (C) Quantification of band intensities from western blots displayed as a triplot of mean glycoform ratios ± standard deviations for each group. Arrows indicate the axis to be read from points to determine the percent of each glycoform. The glycoform ratio of D10 p1 (open circle) is not significantly different from the original D10 ratio (black square), while the D10 p2 ratio is significantly different (black circle). sPMCA of D10 (open diamonds) produces material displaying a glycoform ratio closely resembling both D10 and D10 p1. Passage of sPMCA D10 into Tg(cerPrP)1536 mice results in a further shift towards monoglycosylated PrP^{CWD} (black diamonds), differing significantly from D10 and resembling D10 p2. RML (black triangles) exhibited the greatest proportion of monoglycosylation and was significantly different than all other strains. (D) Triplot of raw data of at least six samples for each group used to generate the mean glycoforms ratios in (C).

sPMCA and mouse passage of D10 changes PrP^{CWD} conformational stability

We further characterized these prion inocula biochemically by assessing conformational stability in the presence of GdnHCl, another established criteria for prion strain typing[30,31,43,46]. We quantified PrP^{RES} band intensities from western blots of brain extracts incubated with increasing GdnHCl concentrations then PK to determine the concentration of GdnHCl required to PK digest 50% of PrP^{RES} ($[GdnHCl]_{50}$). D10, mouse-passaged D10 (D10 p1) and serially-amplified D10 (sPMCA) exhibited nearly identical conformational stabilities, with mean $[GdnHCl]_{50}$ values of 2.35 ± .01, 2.35 ± .10 and 2.33 ± 0.12 M, respectively (Figure 6). Conformational stability was significantly reduced upon secondary passage of D10 (D10 p2, 2.03 ± 0.06 M, $p < 0.01$) and primary passage of amplified D10 (Amp D10 p1, 1.89 ± 0.06 M, $p < 0.01$) in Tg(cerPrP)1536. The RML prion strain was significantly less stable than all other strains (1.46 ± 0.01 M, $p < 0.01$). *In toto*, the biological, neuropathological and biochemical data reported here

strongly support the conclusion that D10 strain adaptation has occurred *in vitro* and *in vivo*.

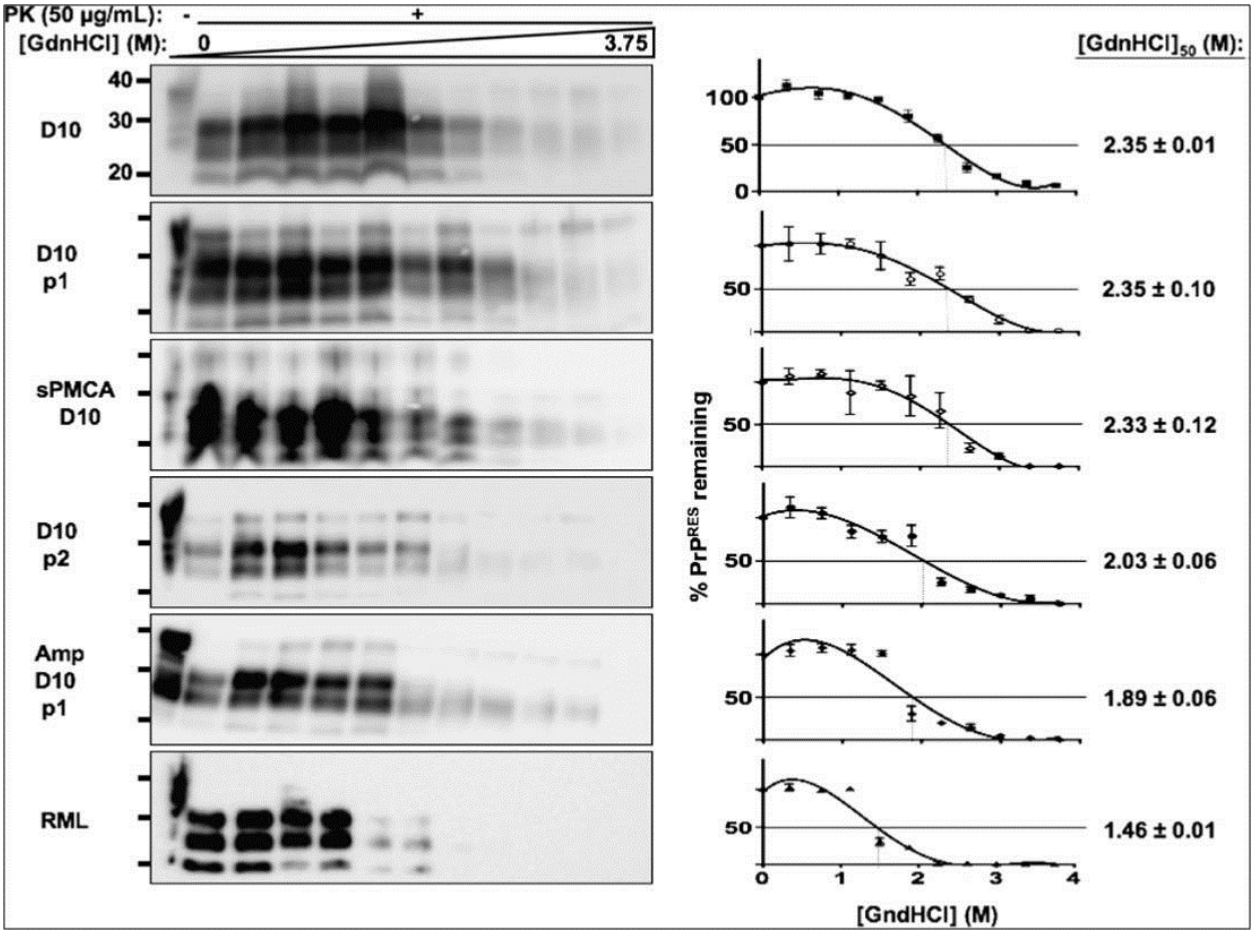


Figure 2.6. Strain adaptation changes PrP^{CWD} conformational stability. Western blots of samples from the indicated prion strains treated with increasing concentrations of guanidine hydrochloride (GdnHCl) and 50 µg/mL PK reveal that D10 retains its conformational stability after primary passage in Tg(cerPrP)1536 mice (D10 p1) or serial amplification (sPMCA). Secondary passage (D10 p2) or primary passage of amplified D10 (Amp D10 p1) destabilizes PrP^{CWD} conformation, more closely resembling that of RML. Denaturation curves to the right of each blot quantify PrP^{RES} conformational stability (see Methods). The concentration of GdnHCl required to denature 50% of PrP^{RES} ($[GdnHCL]_{50}$), a measure of the conformational stability, is shown to the right of the corresponding plot. Data are from at least three independent experiments using at least three animals per group.

DISCUSSION

The first demonstration of amplifying PrP^{CWD} using PMCA was recently reported to yield an approximately 200-fold amplification efficiency per round [17]. In this study we have increased this efficiency up to several thousand-fold per round by optimizing NBH preparation and sonication parameters. We also demonstrated the specificity of the reaction by using $moPrP^{C}$ substrates as negative controls. We observed a linear relationship between –fold amplification and D10 dilutions between 10^{-3} and 2×10^{-5}. Limiting substrate at lower dilutions and limiting prion templates at higher dilutions probably account for asymptotic amplification efficiencies. By maintaining PrP^{CWD} concentrations in the dynamic range of PMCA, we were able to produce linear amplification over repeated PMCA rounds. This resulted in highly efficient and reproducible amplification over at least eight PMCA rounds that generated significant amounts of amplified 10^{-24} D10 with which we infected Tg(cerPrP)1536 mice. Comparing equal dilutions of the original D10 inoculum and amplified 10^{-24} D10 by western blot confirmed that both samples displayed nearly identical signal intensities.

We next demonstrated the first *in vitro* generation of infectious CWD prions using amplified 10^{-24} D10 as the inoculum. *In vitro*-amplified D10 elicited terminal prion disease earlier than the original D10 used to seed the sPMCA reaction. This result was surprising because generation of infectious hamster-adapted scrapie prions *in vitro* using PMCA has previously been reported to elicit terminal disease later than the original scrapie inocula, a phenomenon largely abrogated by serial passage or by conjugation of amplified material to nitrocellulose prior to inoculation[12-15]. One possible explanation for this discrepancy is that previous studies used homologous brain inocula,

substrates and hosts, whereas we used infected cervid brain as the prion inocula and Tg(cerPrP)1536 mice as a source for brain substrate and host. Primary passage of cervid-derived D10 into a murine host may decrease prion replication efficiency, which increases upon *in vitro* amplification using NBH from Tg(cerPrP)1536 mice as a substrate or secondary passage in Tg(cerPrP)1536 mice. Indeed, we observed a reduction in mean incubation time to terminal disease upon serial passage of D10 in Tg(cerPrP)1536 mice, in accordance with another recent report [47]. These data implicate other host factors present in the brain that may influence prion amplification *in vitro* and *in vivo*, resulting in nearly identical CWD prion infectivity titres that we observed for sPMCA-amplified and mouse-passaged D10. Many prion-forming cofactors have been proposed, including proteins [48-50], polysaccharides [51,52] or polyanions [53,54] that may facilitate host-specific prion amplification *in vitro* and *in vivo*. Another possibility postulated by Soto and colleagues is that *in vitro*-generated scrapie prions may have created a new prion strain that requires *in vivo* adaptation to acquire the same infectivity titre as the original prion strain [12]. Our data supports this hypothesis, but because sPMCA generated a cervid prion strain using mouse NBH expressing cerPrP^{C}, we conclude that strain adaptation occurred *in vitro*, resulting in a more infectious prion strain in mice. Correlation with our *in vivo* data demonstrating mouse adaptation of D10 following serial passage strongly supports this interpretation. Serial passage of natural sheep scrapie and bovine spongiform encephalopathy prion isolates in Tg(ovPrP) mice has also recently been shown to reduce incubation times [55,56]. As a corollary to the experiments conducted here, it would be interesting to determine whether sPMCA could

be used to adapt natural scrapie isolates *in vitro* and increase their infectivity titres as does *in vivo* adaptation via mouse passage.

Another straightforward explanation for the shortened incubation times we observed is that inocula from *in vitro* amplification and *in vivo* adaptation contains no heterologous brain homogenate present in the original D10 inoculum, abrogating a potential immune response that may clear a significant portion of the initial inocula. Seminal work comparing mouse and hamster prion titres in mice showed that hamster prions, containing heterologous hamster brain homogenate, induced disease much earlier than mouse prions containing homologous mouse brain homogenate, arguing against immune clearance significantly affecting prion titers[4]. Indeed, we have never observed any clinical, cellular, biochemical or molecular evidence of inflammation upon i.c. inoculation with heterologous brain material (our unpublished data), most likely because the brain is a relatively immune privileged site. In the present study, if immune clearance simply lowered the effective D10 prion dose compared to *in vitro*-amplified or mouse-passaged D10 without strain adaptation, then both of these D10-derived strains should exhibit increased infectivity titres while maintaining other strain properties of the original D10. We explored this possibility by first comparing neuropathological properties of the original D10 strain, *in vitro*-amplified and mouse-passaged D10, and RML. Both *in vitro*-amplified and *in vivo*-adapted D10 prions induced similar neuropathology in Tg(cerPrP)1536 mice, characterized by diffuse PrP^{CWD} deposition that is similar to that observed in brains from wild type mice infected with RML, a scrapie strain well-adapted to the mouse [41,57,58]. These three prion strains produced PrP^{CWD} deposition clearly different than the dense, punctate plaques evident in brains of

Tg(cerPrP)1536 mice inoculated with the original D10 strain. Similar diffuse PrP^{RES} deposition has been observed in RML-infected and CWD-infected mice expressing mouse $PrP^{C59,60}$ and scrapie-infected mice expressing bovine and mouse–bovine chimeric PrP^{C31}. Amplified and mouse-passaged D10 and RML also produced similar brain lesion profiles that were consistent with neuropathology previously reported in scrapie-infected mice[58,61,62] and distinct from the profile induced by the original D10 strain. The changes in location and severity of prion lesions evident among these prion strains are consistent with D10 strain adaptation by *in vitro* amplification that is indistinguishable from *in vivo* adaptation via passage of D10 in Tg(cerPrP)1536 mice.

We also detected biochemical similarities among *in vitro* and *in vivo*-adapted prion strains. While sPMCA D10 and D10 p1 displayed nearly identical PrP^{CWD} glycoforms ratios as the parental D10 strain, Amp D10 p1 and D10 p2 significantly increased monoglycosylated PrP^{CWD} compared to the principally diglycosylated D10 strain, similar to the transition observed in another study of murine adaptation of CWD prions[59]. These results are intriguing in light of another recent study demonstrating that host PrP^{C} glycoforms dictate *in vitro* PrP^{Sc} amplification efficiency[63], suggesting that perhaps murine PrP^{C} glycoforms select, from a heterologous mixture, distinct strains of CWD prions that are preferentially amplified *in vitro* or *in vivo*. We also show that Amp D10 p1 and D10 p2 conformations were significantly more destabilized by GdnHCl denaturation compared to sPMCA D10 and D10 p1, the stabilities of which were nearly identical to that of D10. RML adopted a conformation that was by far the least stable of all prion strains investigated. Previous reports have documented relatively less stable mouse-passaged scrapie prion conformations and more stable CWD prion

conformations[31,43,47]. Our data demonstrate that serial passage of CWD prions in Tg(cerPrP)1536 mice can destabilize their conformation, and that sPMCA using Tg(cerPrP) NBH as substrate expedites this process. Interestingly, decreased stability of *in vitro* and *in vivo*-adapted D10 prion strains correlates with shortened incubation times in Tg(cerprP)1536 mice, a phenomenon previously exhibited for other novel prion strains generated *in vitro* [64] and *in vivo* [46].

We did not observe statistically significant changes in either glycosylation or conformational stability of sPMCA D10 or D10 p1, as one might expect if adaptation had occurred. Perhaps sPMCA and primary passage of D10 initiates creation of a new or intermediate prion strain, or selection of a small concentration of a pre-existing one that, upon further passage into mice, is preferentially selected and amplified. This interpretation is supported by the increased variability in the biochemical properties of D10 p1 (glycosylation and conformational stability) and sPMCA (conformational stability), which may indicate the emergence of nascent prion strains.

The similar neuropathological and biochemical characteristics of *in vitro* and *in vivo*-adapted D10 that clearly differentiate them from primary-passaged D10 prions strongly indicate that sPMCA has adapted D10 as effectively as *in vivo* adaptation by mouse passage. However, we cannot rule out the possibility that *in vitro* amplification and *in vivo* passage of D10 also increased the prion titre of these strains, either by strain adaptation, immune abrogation, or both. Unlike RML, whose titre has been determined by mouse bioassay and cell culture assays [65,66], exact titres for most scrapie and all CWD prion strains have yet to be determined. Comparing western blot signal intensities of PrP^{RES} remains a crude estimate of prion infectivity because PrP^{RES}

concentration and prion infectivity do not always closely correlate[44,45]. Here we present evidence for strain adaptation by sPMCA. The possibility of a concomitant increase in prion titre would also be a novel and interesting result of prion amplification by sPMCA.

We conclude that *in vitro* D10 amplification parallels primary passage of D10 in Tg(cerPrP)1536 mice, and that sPMCA represents a viable means of adapting prion strains to new hosts. Since strain adaptation correlates with species barriers, we envision sPMCA to potentially be a powerful tool with which to probe strain differences and assess species barriers in a fraction of the time and cost of animal bioassays.

REFERENCES

1 Prusiner, S. B. Novel proteinaceous infectious particles cause scrapie. *Science* **216**, 136-144 (1982).

2 Oesch, B. *et al.* A cellular gene encodes scrapie PrP 27-30 protein. *Cell* **40**, 735-746 (1985).

3 Hope, J. *et al.* The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). *Embo J* **5**, 2591-2597 (1986).

4 Büeler, H. R. *et al.* Mice devoid of PrP are resistant to scrapie. *Cell* **73**, 1339-1347 (1993).

5 Bolton, D. C., McKinley, M. P. & Prusiner, S. B. Identification of a protein that purifies with the scrapie prion. *Science* **218**, 1309-1311 (1982).

6 Hope, J. *et al.* Fibrils from brains of cows with new cattle disease contain scrapie-associated protein. *Nature* **336**, 390-392 (1988).

7 Collinge, J. *et al.* Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature* **378**, 779-783 (1995).

8 Scott, M. R. *et al.* Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. *Proc Natl Acad Sci U S A* **96**, 15137-15142 (1999).

9 Race, R. E. *et al.* Comparison of abnormal prion protein glycoform patterns from transmissible spongiform encephalopathy agent-infected deer, elk, sheep, and cattle. *J Virol* **76**, 12365-12368 (2002).

10 Browning, S. R. *et al.* Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *J Virol* **78**, 13345-13350 (2004).

11 Legname, G. *et al.* Synthetic mammalian prions. *Science* **305**, 673-676 (2004).

12 Castilla, J., Saa, P., Hetz, C. & Soto, C. In vitro generation of infectious scrapie prions. *Cell* **121**, 195-206 (2005).

13 Deleault, N. R., Harris, B. T., Rees, J. R. & Supattapone, S. Formation of native prions from minimal components in vitro. *Proc Natl Acad Sci U S A* **104**, 9741-9746 (2007).

14 Weber, P. *et al.* Generation of genuine prion infectivity by serial PMCA. *Vet Microbiol* **123**, 346-357 (2007).

15 Murayama, Y. *et al.* Urinary excretion and blood level of prions in scrapie-infected hamsters. *J Gen Virol* **88**, 2890-2898 (2007).

16 Murayama, Y. *et al.* Efficient in vitro amplification of a mouse-adapted scrapie prion protein. *Neurosci Lett* **413**, 270-273 (2007).

17 Kurt, T. D. *et al.* Efficient in vitro amplification of chronic wasting disease PrPRES. *J Virol* **81**, 9605-9608 (2007).

18 Hadlow, W. J. Scrapie and kuru. *Lancet* **2**, 289-290 (1959).

19 Gajdusek, D. C., Gibbs, C. J. & Alpers, M. Experimental transmission of a Kuru-like syndrome to chimpanzees. *Nature* **209**, 794-796 (1966).

20 Gibbs, C. J., Jr. & Gajdusek, D. C. Transmission and characterization of the agents of spongiform virus encephalopathies: kuru, Creutzfeldt-Jakob disease, scrapie and mink encephalopathy. *Res Publ Assoc Res Nerv Ment Dis* **49**, 383-410 (1971).

21 Taraboulos, A. *et al.* Regional mapping of prion proteins in brain. *Proc Natl Acad Sci U S A* **89**, 7620-7624 (1992).

22 Bessen, R. A. & Marsh, R. F. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *J Virol* **68**, 7859-7868 (1994).

23 Telling, G. C. *et al.* Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity *Science* **274**, 2079-2082 (1996).

24 Korth, C. *et al.* Abbreviated incubation times for human prions in mice expressing a chimeric mouse-human prion protein transgene. *Proc Natl Acad Sci U S A* **100**, 4784-4789 (2003).

25 Safar, J., Roller, P. P., Gajdusek, D. C. & Gibbs, C. J., Jr. Thermal stability and conformational transitions of scrapie amyloid (prion) protein correlate with infectivity. *Protein Sci* **2**, 2206-2216 (1993).

26 Collinge, J., Sidle, K. C., Meads, J., Ironside, J. & Hill, A. F. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383**, 685-690 (1996).

27 Peretz, D. *et al.* A conformational transition at the N terminus of the prion protein features in formation of the scrapie isoform. *J Mol Biol* **273**, 614-622 (1997).

28 Hill, A. F. *et al.* The same prion strain causes vCJD and BSE [letter] *Nature* **389**, 448-450 (1997).

29 Safar, J. *et al.* Eight prion strains have PrP(Sc) molecules with different conformations *Nat Med* **4**, 1157-1165 (1998).

30 Peretz, D. *et al.* Strain-specified relative conformational stability of the scrapie prion protein. *Protein Sci* **10**, 854-863 (2001).

31 Scott, M. R., Peretz, D., Nguyen, H. O., Dearmond, S. J. & Prusiner, S. B. Transmission barriers for bovine, ovine, and human prions in transgenic mice. *J Virol* **79**, 5259-5271 (2005).

32 Collinge, J. & Rossor, M. A new variant of prion disease. *Lancet* **347**, 916-917 (1996).

33 Will, R. G. *et al.* A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347**, 921-925 (1996).

34 Raymond, G. J. *et al.* Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. *Embo J* **19**, 4425-4430. (2000).

35 Collinge, J. *et al.* Transmission of fatal familial insomnia to laboratory animals. *Lancet* **346**, 569-570 (1995).

36 Hamir, A. N. *et al.* Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. *J Vet Diagn Invest* **17**, 276-281 (2005).

37 Hamir, A. N. *et al.* Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation. *J Vet Diagn Invest* **18**, 558-565 (2006).

38 Hamir, A. N., Kunkle, R. A., Miller, J. M., Bartz, J. C. & Richt, J. A. First and second cattle passage of transmissible mink encephalopathy by intracerebral inoculation. *Vet Pathol* **43**, 118-126 (2006).

39 Hamir, A. N. *et al.* Preliminary observations on the experimental transmission of scrapie to elk (Cervus elaphus nelsoni) by intracerebral inoculation. *Vet Pathol* **40**, 81-85 (2003).

40 Fischer, M. *et al.* Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* **15**, 1255-1264 (1996).

41 Prusiner, S. B., Hadlow, W. J., Eklund, C. M., Race, R. E. & Cochran, S. P. Sedimentation characteristics of the scrapie agent from murine spleen and brain. *Biochemistry* **17**, 4987-4992 (1978).

42 Fraser, H. & Dickinson, A. G. The sequential development of the brain lesion of scrapie in three strains of mice. *J Comp Pathol* **78**, 301-311 (1968).

43 Xie, Z. *et al.* Chronic wasting disease of elk and deer and Creutzfeldt-Jakob disease: comparative analysis of the scrapie prion protein. *J Biol Chem* **281**, 4199-4206 (2006).

44 Hill, A. F., Antoniou, M. & Collinge, J. Protease-resistant prion protein produced in vitro lacks detectable infectivity. *J Gen Virol* **80 (Pt 1)**, 11-14 (1999).

45 Rubenstein, R. *et al.* Concentration and distribution of infectivity and PrPSc following partial denaturation of a mouse-adapted and a hamster-adapted scrapie strain. *Arch Virol* **139**, 301-311 (1994).

46 Peretz, D. *et al.* A change in the conformation of prions accompanies the emergence of a new prion strain. *Neuron* **34**, 921-932 (2002).

47 Green, K. M. *et al.* The elk PRNP codon 132 polymorphism controls cervid and scrapie prion propagation. *J Gen Virol* **89**, 598-608 (2008).

48 Telling, G. C. *et al.* Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* **83**, 79-90 (1995).

49 Kaneko, K. *et al.* Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. *Proc Natl Acad Sci U S A* **94**, 10069-10074 (1997).

50 Yehiely, F. *et al.* Identification of candidate proteins binding to prion protein. *Neurobiol Dis* **3**, 339-355 (1997).

51 Appel, T. R., Dumpitak, C., Matthiesen, U. & Riesner, D. Prion rods contain an inert polysaccharide scaffold. *Biol Chem* **380**, 1295-1306 (1999).

52 Dumpitak, C. *et al.* The polysaccharide scaffold of PrP 27-30 is a common compound of natural prions and consists of alpha-linked polyglucose. *Biol Chem* **386**, 1149-1155 (2005).

53 Deleault, N. R. *et al.* Post-transcriptional suppression of pathogenic prion protein expression in Drosophila neurons. *J Neurochem* **85**, 1614-1623 (2003).

54 Deleault, N. R., Lucassen, R. W. & Supattapone, S. RNA molecules stimulate prion protein conversion. *Nature* **425**, 717-720 (2003).

55 Bencsik, A. *et al.* Scrapie strain transmission studies in ovine PrP transgenic mice reveal dissimilar susceptibility. *Histochem Cell Biol* **127**, 531-539 (2007).

56 Baron, T. & Biacabe, A. G. Molecular behaviors of "CH1641-like" sheep scrapie isolates in ovine transgenic mice (TgOvPrP4). *J Virol* **81**, 7230-7237 (2007).

57 Chandler, R. L. Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* **1**, 1378-1379 (1961).

58 Kingsbury, D. T. *et al.* Genetic control of scrapie and Creutzfeldt-Jakob disease in mice. *J Immunol* **131**, 491-496 (1983).

59 Sigurdson, C. J. *et al.* Strain fidelity of chronic wasting disease upon murine adaptation. *J Virol* **80**, 12303-12311 (2006).

60 Zabel, M. D. *et al.* Stromal complement receptor CD21/35 facilitates lymphoid prion colonization and pathogenesis. *J Immunol* **179**, 6144-6152 (2007).

61 Eklund, C. M., Kennedy, R. C. & Hadlow, W. J. Pathogenesis of scrapie virus infection in the mouse. *J Infect Dis* **117**, 15-22 (1967).

62 Baringer, J. R. & Prusiner, S. B. Experimental scrapie in mice: ultrastructural observations. *Ann Neurol* **4**, 205-211 (1978).

63 Nishina, K. A. *et al.* The stoichiometry of host PrPC glycoforms modulates the efficiency of PrPSc formation in vitro. *Biochemistry* **45**, 14129-14139 (2006).

64 Legname, G. *et al.* Strain-specified characteristics of mouse synthetic prions. *Proc Natl Acad Sci U S A* **102**, 2168-2173 (2005).

65 Klohn, P. C., Stoltze, L., Flechsig, E., Enari, M. & Weissmann, C. A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. *Proc Natl Acad Sci U S A* **100**, 11666-11671 (2003).

66 Prusiner, S. B. *et al.* Measurement of the scrapie agent using an incubation time interval assay. *Ann Neurol* **11**, 353-358 (1982).

CHAPTER 3:

DE NOVO GENERATION OF INFECTIOUS CERVID PRIONS USING PROTEIN MISFOLDING CYCLIC AMPLIFICATION (PMCA)

SUMMARY

Chronic wasting disease is a prion disease that affects free ranging and captive cervids (deer, elk, and moose). Substantial evidence suggests that prions are misfolded, infectious, insoluble, and protease resistant proteins (PrP^{RES}) devoid of nucleic acid. Protein misfolding cyclic amplification (PMCA) has provided additional evidence that PrP^{RES} acts as a template that can convert normal prion protein (PrP^{C}) into the infectious misfolded PrP^{RES} isoform. Human PrP^{C} has been shown to rarely spontaneously convert into an infectious misfolded state causing sporadic Creutzfeldt-Jakob disease (sCJD). Recently, several investigators have reported spontaneous generation of prions utilizing *in vitro* methods, including PMCA. Here we tested the rate of *de novo* generation of prions in our laboratory using our standard PMCA protocol. We report that we were able to generate *de novo* prions in rounds 4,5, and 7 at low rates of 1.6, 5.0, and 6.7% respectively. The prions were infectious upon inoculation into cervidized mice and they displayed similar but unique characteristics to other cervid prion strains.

INTRODUCTION

Prions cause diseases classified as transmissible spongiform encephalopathies (TSEs) which are characterized by distinct neuropathologic vacuoles and accumulation of a transmissible proteinase K (PK)-resistant protein (PrP^{RES}) [1,2,3]. Bovine spongiform encephalopathy (BSE) of cattle, Creutzfeldt-Jakob disease (CJD) of humans, scrapie of

sheep and goats, and chronic wasting disease (CWD) of cervids (deer, elk and moose) are notable prion diseases that can be transmitted, inherited, or occur spontaneously. Mounting evidence demonstrates that prion pathogenesis is caused by the conversion of the normal cellular host protein, (PrP^{C}) into a protease-resistant, abnormal disease-causing isoform devoid of nucleic acid (PrP^{RES}) [4,5, 6,7,8].

In vitro generation of infectious prion protein using protein misfolding cyclic amplification (PMCA) has substantiated the protein-only hypothesis. PMCA utilizes sonication and incubation steps to break up PK-resistant aggregates encouraging the prion seed to interact and template PrP^{C} present in the uninfected brain homogenate substrate[9]. Employing repeated cycles of incubation and sonication has led to efficient amplification of minute quantities of PrP^{RES} using substrate from varied species[9-13]. Serial PMCA (sPMCA) has also been employed to successfully evaluate strain adaptation and species barriers in the absence of lengthy and expensive bioassays[10,14-16]. Intriguingly, Deleault et al., interrogated PMCA component requirements for *in vitro* amplification resulting in spontaneous generation of infectious prions from non-infectious components: native hamster PrP^{C} in combination with co-purified lipids and synthetic polyanions[17]. Barria et al., expanded on these findings and reported generating spontaneous prions from uninfected normal brain homogenate substrate in a prion free laboratory upon extended rounds and modification of normal sPMCA conditions[18].

In order to examine the rate of spontaneous conversion with our PMCA protocol we subjected uninfected brain homogenate derived from cervidized transgenic mice to seven rounds (48 cycles each) of PMCA under normal conditions. In order to avoid the

possibility of cross contamination all experiments were performed in a prion free lab with new reagents and new equipment. Surprisingly, we were able to detect PK-resistant bands by western blot at round 4 of sPMCA at a rate of 1.6%. Further generation of spontaneous prions were observed after round 5 and round 7 with rates of 5.0% and 6.74% respectively. Bioassay determined that the spontaneously generated cervid prions were infectious to cervidized transgenic but not wild type mice and biochemical analysis resulted in a unique profile differing from prion strains used within our laboratory. These data strongly suggest that these prions were derived spontaneously and not from contamination. Recent evidence suggests that amino acids including and between 170 and 174 of the cervid prion protein structurally form a rigid loop increasing the propensity for misfolding[19-23]. We propose that the *de novo* generation of infectious cervid prions in our laboratory is a novel cervid prion strain that occurred spontaneously with the aid of PMCA and occurs at a very low frequency after 8 rounds of 48 cycles of sPMCA.

MATERIALS AND METHODS

Preparation of normal brain homogenate (NBH)

All NBH preparations were completed in a prion free laboratory not in association with any of the prion laboratories in the building. Mice were euthanized and perfused with 30 ml 5mM EDTA in PBS. Whole brains were removed and immediately frozen in liquid nitrogen. Brains were weighed and transferred into 1.5 mL Eppendorf tubes with 2.5 mm glass beads. PMCA buffer with 2X Complete Protease Inhibitor Cocktail (Roche) was added to make a 20 % w/v solution. Samples were homogenized for 20 s at 4.5 m/s in a FastPrep machine (Biogene), cooled on ice for two minutes and

centrifuged at 14,000 x g for 10 s to reduce foaming. This process was repeated twice. NBH was diluted to a 10% w/v solution by adding an equal volume of PMCA conversion buffer containing 2% Triton X-100 and incubated on ice for 20 min. NBH was clarified by centrifuging for 5 min at 1,500 x g and supernatants were aliquoted to into new tubes and stored at -70^{o}C.

Serial PMCA

Serial PMCA (sPMCA) was conducted in a new prion free laboratory separate from the laboratory the NBH was made. All reagents and equipment were new and never used in a prion contaminated laboratory. sPMCA was modified from a previous protocol (Appendix A1.6)[15]. Briefly, 50 µL samples of 10% NBH were placed into 20 wells of a 96-well microplate. The entire plate was suspended in the water bath of a new 3000MP sonicator (Misonix) and sonicated at 70- 85% maximum power for 40 seconds, followed by 30-minute incubation at 37^{O}C. This cycle was repeated 48 times constituting 1 round. For each additional round 25 µL of NBH from the previous round was diluted into 25 µL of fresh NBH and subjected to another PMCA round. This process was repeated for a total of 8 rounds. Replicate experiments of 20 NBH samples were started 3 days from each other totalling in 3 experiments and 60 NBH samples. Gloves were changed between each replicate group and samples were western blotted immediately after the completed round to look for PK resistant material. Any PK resistant material that was definitively positive was not subjected to further rounds of PMCA to avoid cross contamination of other samples. Those samples that were not definitively positive (low band intensities and banding patterns that mimicked undigested material) were

subjected to another round of PMCA to confirm positivity. If they also showed positivity at the next round they were called positive at the previous round.

Mice

B6129SF2/J mice (stock number 101045) were purchased from the Jackson Laboratories (Bar Harbor, ME). Tg(cerPrP)5037 were generated as previously described[24]. All mice were bred and maintained at Lab Animal Resources, accredited by the Association for Assessment and Accreditation of Lab Animal Care International, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University.

Sources and preparation of prion inocula

10% brain homogenates were prepared in PMCA buffer (4mM EDTA, 150 mM NaCl in PBS). PMCA samples that were western blot positive indicating *de novo* prions were pooled together to be used as inoculum. We diluted equal volumes of the inoculum 1:10 in 320 mM sucrose supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (Gibco) in PBS thirty minutes prior to intracerebral inoculations. Remaining *de novo* brain homogenate from PMCA was further amplified to 10 rounds in order to create enough *de novo* positive brain material for biochemical analysis.

PK digestion and western blotting

Samples were digested with 50 µg/ml PK (Roche) for 30 min at 37^{o}C. The reaction was stopped by adding lithium dodecyl sulfate sample loading buffer (Invitrogen) and incubating at 95^{o}C for 5 min. Proteins were electrophoretically separated through 12% sodium dodecyl sulfate-polyacrylamide gels (Invitrogen), and transferred to polyvinylidene difluoride membranes (Millipore). Non-specific membrane

binding was blocked by incubation in 5% milk blocking solution (Bio-Rad) for 1 h. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated Bar224 anti-PrP monoclonal antibody (SPI bio) diluted 1:20,000 in Superblock (Pierce), washed 6 x 10 min in PBS with 0.2% Tween 20, and incubated for 5 min with enhanced chemiluminescent substrate (Millipore). Membranes were digitally photographed using the FujiDoc gel documentation system equipped with a cooled charge-coupled diode camera (Fuji). Densitometric analyses were performed using Quantity One software (Bio-Rad).

Prion inoculations and clinical scoring

Mice were anesthetized by Isofluorane inhalation. Thirty microliters of the inoculum was injected intracerebrally 3 mm deep through the coronal suture 3-5 mm lateral of the sagittal suture. Mice were monitored daily for clinical symptoms of prion disease, including tail rigidity, impaired extensor reflex, akinesia, tremors, ataxia, 15 % weight loss and paralysis. Mice with any four of these symptoms were scored terminally sick and euthanized.

Histochemistry and immunohistochemistry (IHC)

Tissues were fixed in 10% paraformaldehyde, embedded in paraffin and 5-10 µm sections mounted on glass slides. For PrP staining tissue sections were deparaffinized, treated with concentrated formic acid for thirty minutes, then autoclaved at 121^{o}C in target retrieval solution (Dako) for 2 h, washed 2 x 7 min in 1X PBS, treated with 0.3% H_20_2 in methanol for 30 min and blocked for 1 hr with 5% BSA in PBS and mixed 1:1 in superblock (Pierce). Excess block was tapped off and sections were incubated with anti-PrP Bar 224 monoclonal antibody diluted 1:500 in block solution for 1 hour. Slides

were then washed 2 x 7 min in PBS and incubated 30 min with Envision+HRP mouse secondary (DAKO). After another 2 x 7 min wash, slides were incubated for 5-7 min with AEC+Substrate-Chromagen (DAKO) and rinsed 2 x 7 min in PBS and counterstained with hematoxylin. Slides were rinsed in H2O, immersed in a 0.1% sodiumbicarbonate bluing reagent for 5 min, rinsed in tap water and coversliped with aqueous mounting medium (Richard Allan Scientific). Hematoxylin and Eosin (H&E) and glial fibrillary acidic protein (GFAP) staining was performed on a NexES automated IHC stainer (Ventana Medical systems, Inc. Tucson, AZ). Sections were stained with H&E for 4 min at room temperature. Sections were stained with rabbit polyclonal antisera against GFAP (diluted 1: 8) for 10 min at 37^{o}C followed by Biotinylated goat anti-rabbit Ig (mouse/rat adsorbed) for 8 min, then counterstained with hematoxylin for 4 min. Sections were visualized and digitally photographed using an Olympus BX60 microscope equipped with a cooled charge-coupled diode camera.

Glycoform Ratios

Western blots of 10% brain homogenates of infected mice were analyzed by densiometric analysis (Quantity One). Di, mono, and unglycosylated banding intensities were calculated as percentages of the total density of each PK treated sample. The averages of 2 replicate samples were plotted on a tri-plot based on calculated glycoform ratios.

Conformational stability assay

Conformational stability assays were modified from a previous protocol[15]. 15 μl aliquots of brain homogenates were incubated for 1 h at room temperature with increasing concentrations of guanidine hydrochloride (GdnHCl, Sigma) ranging from 0

to 5 M in 0.5 M increments. Samples were precipitated in ice-cold methanol at -20^{o}C overnight, and centrifuged at 13,000 X g for 30 min at 4 ^{o}C. Pellets were washed in PMCA buffer and centrifuged 3x, resuspended in 18 μl of PMCA buffer, PK-digested and western blotted. Conformational stability was quantified by densitometric analyses of western blots, plotting the mean percentage of PrP^{RES} remaining ± SD as a function of GdnHCl concentration, and using fourth order polynomial equations and nonlinear regression (GraphPad Prism) to fit denaturation curves for each prion strain.

Lesion profiling

Brain lesion profiling was performed as previously described[15] with slight modifications. Ten neuroanatomic regions were identified in coronal brain sections from at 5 mice of each group: 1-dorsal medulla, 2-cerebellum, 3- superior colliculus, 4-reticular formation, 5-hypothalamus, 6-hippocampus, 7-thalamus, 8-cerebral cortex, 9-primary somatosensory cortex and 10-caudate-putamen. A professional pathologist blinded to the group identification scored each region for vacuolation, astrogliosis and PrP^{RES} deposition using the following severity scale: normal (0), mild (1), moderate (2) severe (3). The average of the sum of the three scores constitutes the severity score for each region.

Statistical analyses

One-way ANOVA with Tukey post-test analysis was performed using GraphPad Prism.

RESULTS

Generation of de novo prions using PMCA

Firstly, we have previously reported that Tg(5037) normal brain homogenate (NBH) supports efficient PMCA amplification using a standard PMCA protocol[15,25,26].

Importantly, false positives in our negative controls have been rare and even under strict measures, contamination cannot be dismissed. Interestingly, the rare occurrence of false positives in our negative controls are primarily observed after 5 rounds of PMCA and are not reproducible while true positives are generally observed by 3 rounds in multiple replicates. To determine the rate of PMCA false positives in our laboratory, we ran Tg(5037) cervidized mouse[24] NBH through our standard sPMCA protocol. Three groups of 20 NBH samples were subjected to 8 rounds of sPMCA in a prion free laboratory to avoid cross-contamination. Additionally, all reagents and equipment were new and brain homogenate was made in a different laboratory unexposed to prions. After each round of PMCA (48 cycles of 30 min incubation followed by 40 sec sonication pulse) NBH samples were analyzed for positivity by western blot and any confirmed positives were removed from the PMCA plate to avoid contamination of the remaining negative samples.

Western blot analysis resulted in identification of one protease-resistant PrP (PrP^{RES}) sample after 4 rounds of sPMCA generating *de novo* PrP^{RES} at a rate of 1.6% (Figure 3.1). sPMCA rounds 5 and 7 propagated *de novo* material accumulating rates of 5.0% and 6.7% respectively (Figure 3.1B).

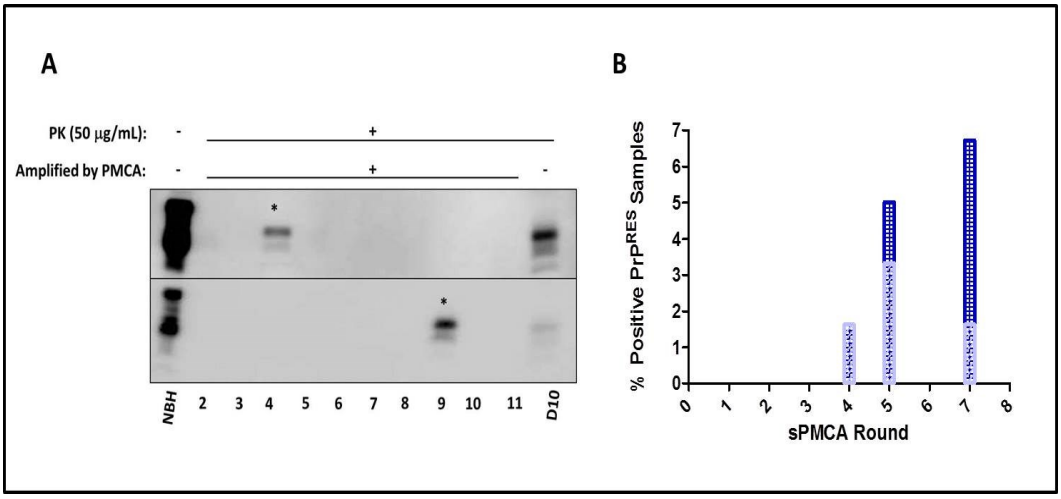


Figure 3.1. (A) Representative western blots of *de novo* prions generated by PMCA. First well is a non-amplified, non-PK treated NBH followed by 10 amplified, PK-treated NBH samples and a non-amplified PrP^{RES} control for comparison. *De novo* prions are indicated by an *. **(B)** Graphical representation of the rate of *de novo* generated prions at respective rounds. The light blue bars indicate the rate of *de novo* generation for that individual round (1.6, 3.3, and 1.6% respectively) and dark blue bars indicate the cumulative rate of *de novo* generation for all rounds (1.6, 5.0, and 6.7% respectively).

De novo prions are infectious

To determine if *de novo* generated prions were infectious, we intracerebrally (i.c.) inoculated cervid transgenic mice (Tg(5037)) and wild type mice with western blot positive *de novo* prions generated by PMCA. Here we report that Tg(5037) mice but not wild type inoculated with *de novo* prions displayed clinical signs of disease. Tg(5037) mice loss 15% of body weight, had a stiff tail, were ataxic, were observed with hind legs splayed behind them when not in motion, but did not exhibit a hind leg grasp when held up by their tail (Figure 3.2C). Curiously *de novo* prions did not have a complete attack rate as only 4/5 Tg(5037) displayed clinical signs by 317 days post inoculation (dpi) and 0/5 wild type mice showed signs of infectivity (Table 3.1, Figure 3.2). We compared dpi of *de novo* prions to a previously reported natural deer prion isolate (D10) and a well characterized mouse-adapted scrapie strain (RML 5)[15]. Mice inoculated with *de novo*

prions generated a survival curve with a comparable mean of 273 dpi to the D10 cervid strain with mean of 282 dpi (Table 3.1, Figure 3.2A). However, Tg(5037) mice inoculated with *de novo* prions had a significant ($p<0.01$) 104 day delay in disease of as compared to cervidized mice inoculated with a serially amplified PMCA D10 (sPMCA) strain (Figure 3.2B). Additionally, *de novo* prions inoculated into wild type or cervidized mice had a lengthened incubation period when compared to mouse adapted scrapie prions. Interestingly, the dpi range among mice inoculated with *de novo* or D10 prions was rather large in contrast to mice inoculated with adapted prion strains (Table 3.1).

Table 3.1. Summary of inoculation experiments comparing *de novo* incidence and incubation to previously reported prion strains from our laboratory[15].

	Cervid		Wild Type	
Inoculum[a]	**Incidence[b]**	**DPI[c] + SD**	**Incidence**	**DPI + SD**
De Novo	4/5	273±61	0/5	>500[e]
D10*	8/8	285±55	0/5	>500
RML*	ND[d]	ND	6/6	155±6
sPMCAD10*	6/6	169±4[e]	ND	ND

[a] Mice were inoculated intracerebrally.
[b] Incidence= number of terminally sick mice/number of animals inoculated
[c] DPI±SD, days post inoculation to terminal disease ± standard deviation.
[d] No data
[e] $p<0.01$ compared to de novo.
* Previously reported data

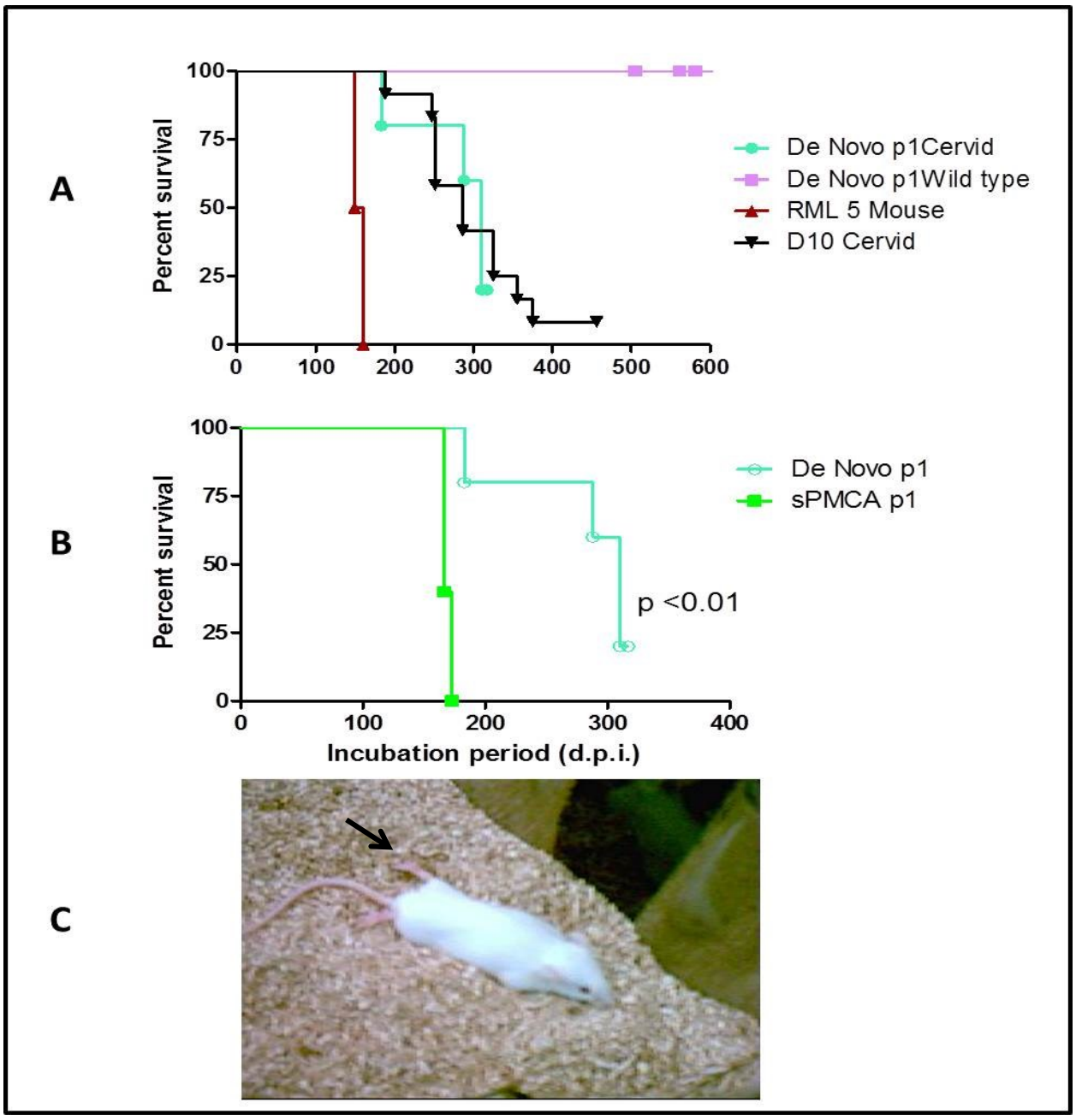


Figure 3.2. Kaplan-Meier survival curves. **(A)** Comparison of *de novo* prions inoculated into Tg(5037) mice resulting in an average of 272 dpi and wild type mice with no disease >500 dpi. Survival curves of D10 and *de novo* were comparable to each other but different (though not significant) from RML prions. **(B)** Comparison of cervidized transgenic mice survival after inoculation with *de novo* prions (*de novop1)* or D10 that was serially amplified by PMCA (sPMCAp1). *De novo* prions displayed an increased incubation period compared to D10 sPMCA prions. **(C)** Representative photo of clinical signs observed in Tg(5037) mice inoculated with *de novo* prions. Arrow points to splaying of hind limbs.

De novo glycoform ratios trend towards a unique phenotype upon inoculation

We further assessed differences of *de novo* generated prions compared to other laboratory prion strains. Glycoform ratios have been a useful tool for characterizing different prion strains as evidenced by a predominate di-glycosylated band in CWD prions as compared to a predominant mono-glycosylated band of scrapie prions[27]. We compared di, mono, and un-glycosylated bands of PK digested brain homogenate from *de* novo generated prions, *de novo* prions passaged through Tg(5037) mice, D10 and RML prions by deniometric analysis of western blot (Figure 3.3A). Mouse number 3 was not analyzed because this mouse did not show signs of clinical disease at time of termination (317 dpi) and was confirmed negative by western blot (Figure 3.3A, lanes 5 and 6). Glycoform ratio averages of *de novo* inoculated mice were plotted on a tri-plot for easy comparison (Figure 3.3B). *De novo* PMCA generated prions exhibited a high ratio of di-glycosylated prions nearly identical to D10. Upon passage of *de novo* prions into Tg(5037) mice, glycoform ratios trended toward an equal ratio of di and mono-glycosylated forms reminiscent RML glycoform ratios but was most comparable to D10 and the original *de novo* inoculum.

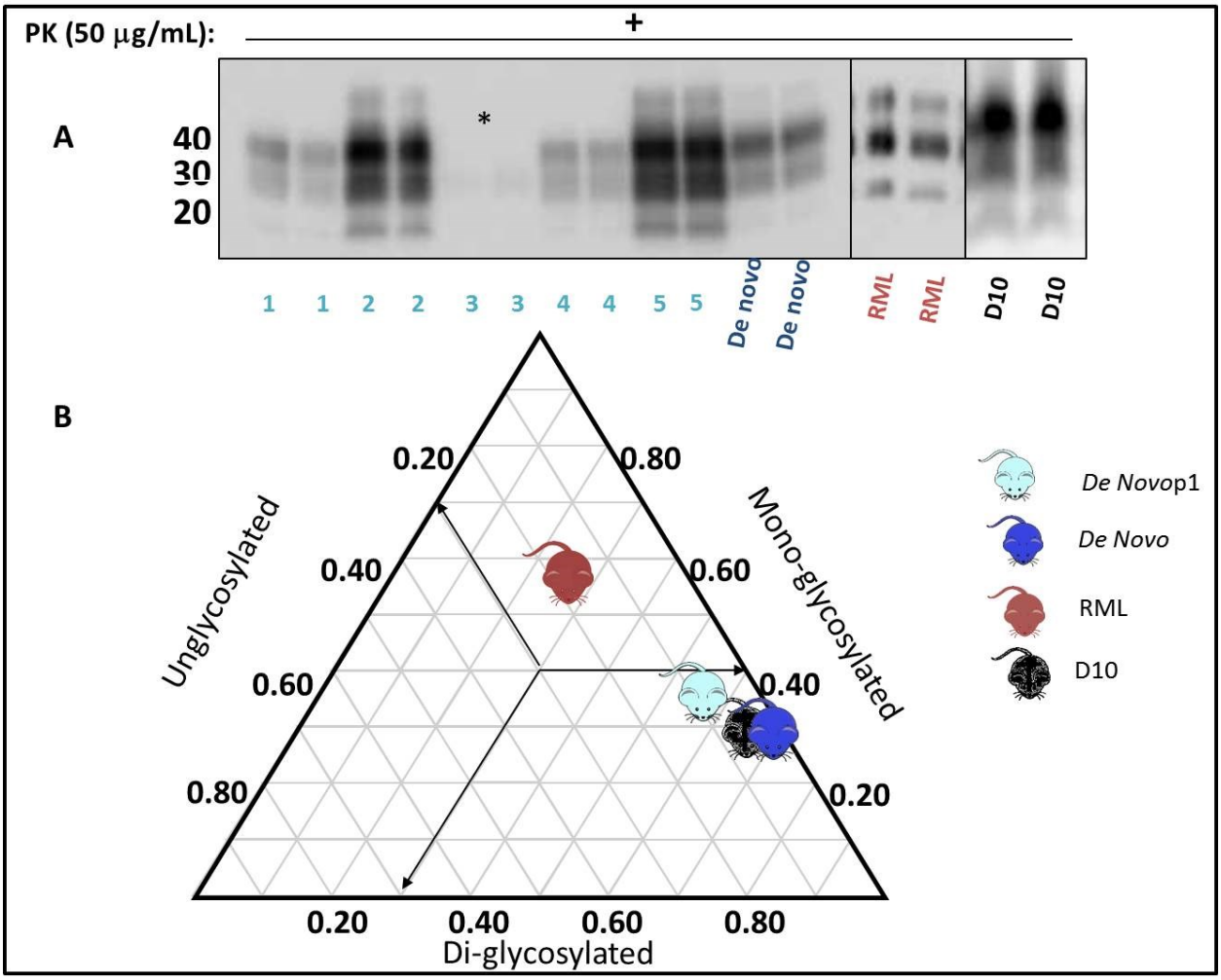


Figure 3.3. (A) Western blot of Tg(5037) brain homogenate of *de novo* inoculated mice (lanes 1-10), *de novo* inoculum (lanes 11,12), RML and D10 from different blots (lanes 15-18). Numbers 1-5 indicate the mouse number. Duplicate samples were analyzed after PK digestion and glycoform ratios were determined by densiometric analysis and averaged together. * indicates non-infected mouse and was not included in the analysis. **(B)** Average glycoform ratios were ploted on a tri-plot arrows indicate the direction of the axis for each glycosylation type. *De novo* prions (dark blue) and D10 prions (black) were predominately di-glycosylated while inoculated Tg(5037) mice (*de novo*p1, turquois) trended toward an equal ratio of di and mono-glycosylation. RML prions (red) had a unique mono-glycosylation pattern as compared to other strains.

De novo prions are stabilized after inoculation

We further assessed *de novo* strain characteristics biochemically by denaturing prion positive brain homogenate with increasing concentrations of guanidine hydrochloride (GdnHCl), followed by PK digestion and western blot analysis of

remaining PrP^{RES} (Figure 3.4A). *De novo* prions exhibited a conformational stability similar to D10 prions propagated through cervidized mice (D10p1) or serially amplified by PMCA (sPMCA) with mean $[GdnHCl]_{50}$ values of 2.25±0.01, 2.35±0.01, and 2.33±0.01 respectively but was significantly different ($p<0.001$) from D10 (Figure 3.4B,C). Passage of *de novo* prions through Tg(5037) mice (*de novo*p1) resulted in a significantly higher ($p<0.001$) $[GdnHCl]_{50}$ value of 3.40±0.01 compared to all strains including, sPMCA prions passaged into cervidized mice (sPMCA p1). Interestingly, sPMCAp1 prions created similarly to *de novo*p1 prions (but with a PMCA seeded reaction), exhibited a much lower $[GdnHCl]_{50}$ value of 1.89±0.01, nearly identical to RML.

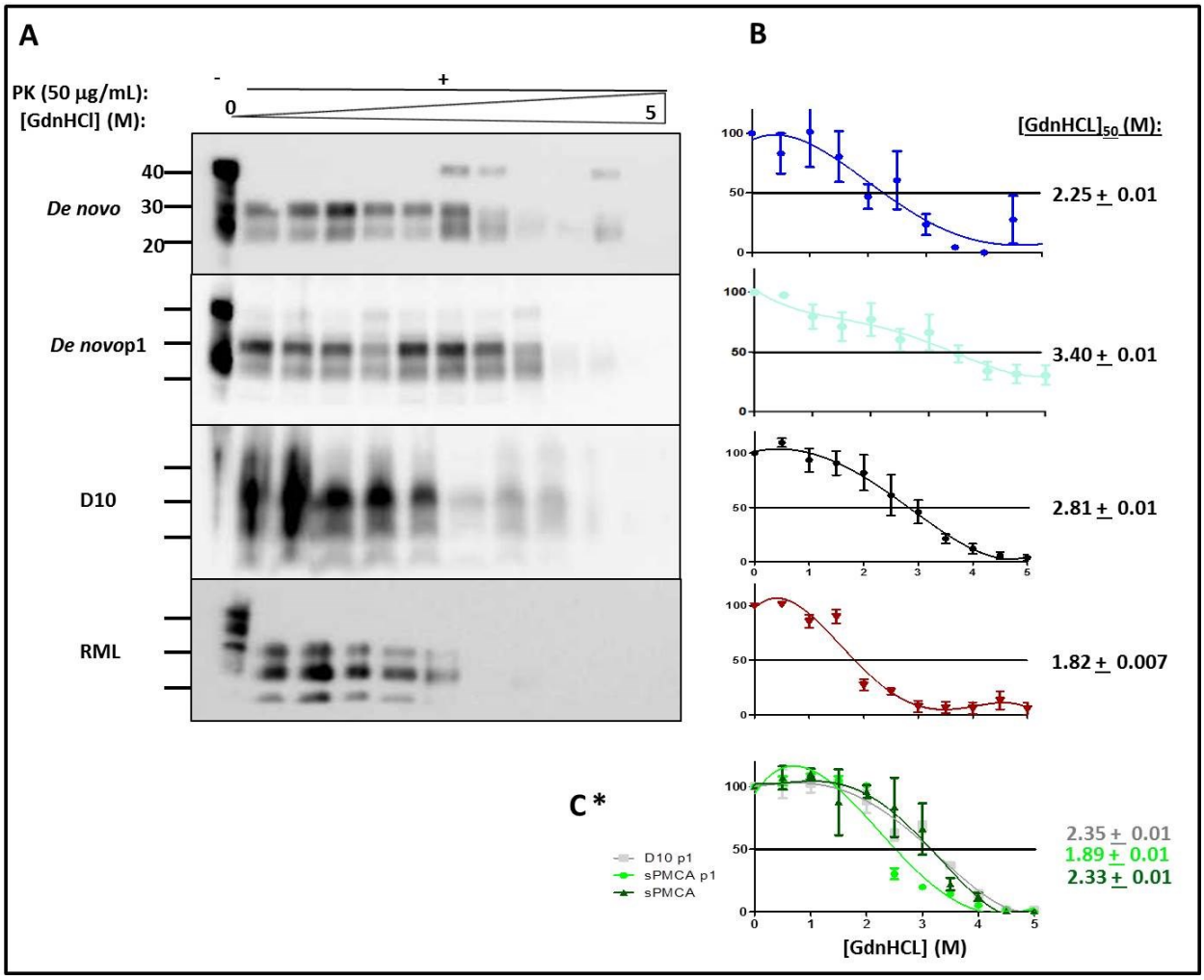
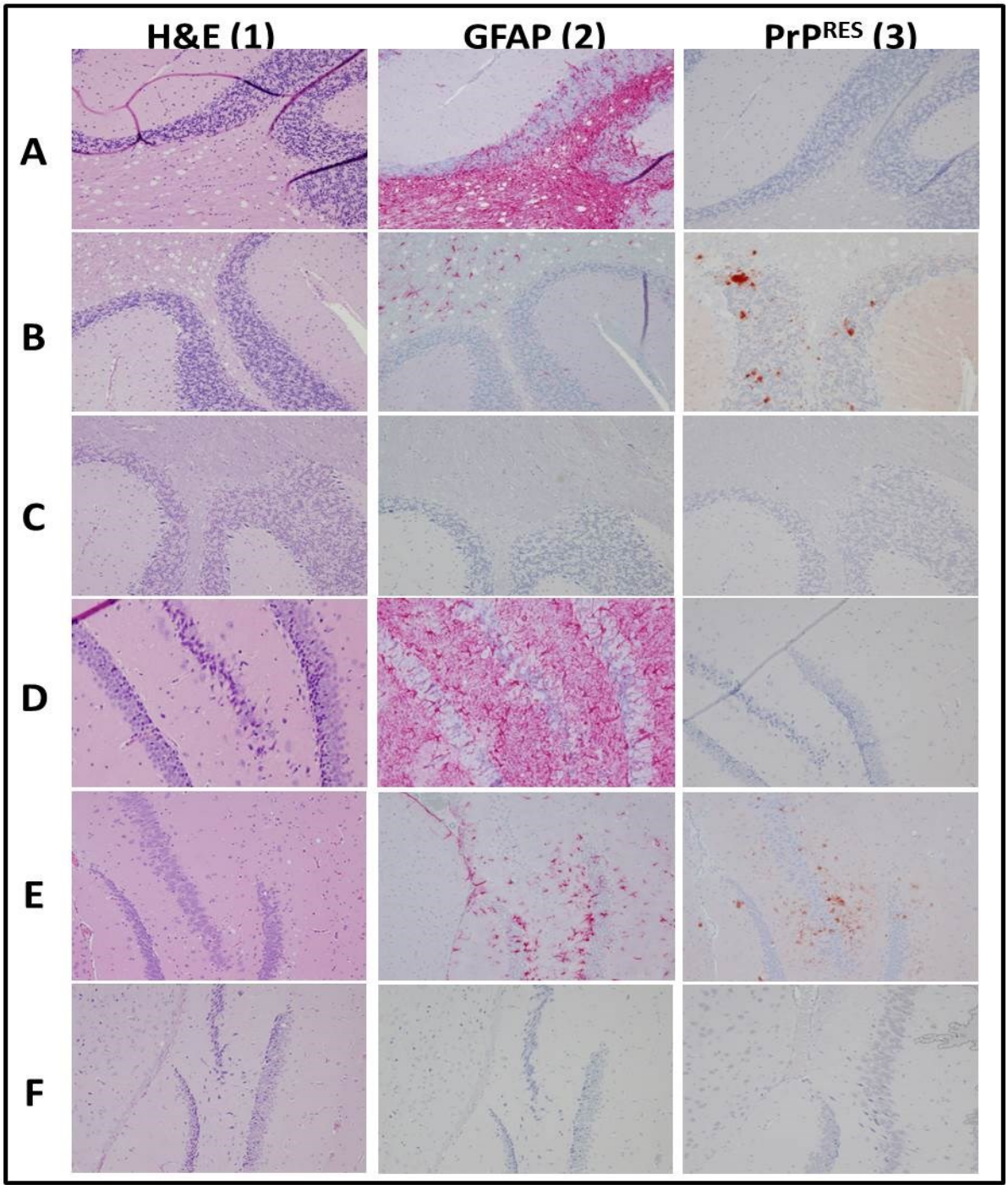


Figure 3.4. (A) Western blots of brain extracts denatured with increasing guanidine hydrochloride (GdnHCl) concentrations and treated with 50µg/ml PK. **(B)** Corresponding denaturation curves quantify remaining PrP^{RES}. To the right of each curve is the concentration of GdnHCl required to denature 50% of the PrP^{RES} ($[GdnHCl]_{50}$). *De novo* prions exhibited a similar conformational stability to D10 prions serially amplified or passaged into cervidized mice but were statistically different from D10. Passage of *de novo* prions into cervidized mice stabilized the conformational stability with a robust ($[GdnHCl]_{50}$ that was not comparable to any other prion strain. **(C*)** Previously reported conformational stability assay results used to additionally compare *de novo* prions.

De novo prions exhibits unique neuropathology after inoculation

Next we assessed vacuolation, astrogliosis, and PrP^{RES} deposition in brain areas of *de novo* prions inoculated into wild type and Tg(5037) mice and compared them to mice inoculated with D10 and RML (Figure 3.5). Brains of wild type mice inoculated with

de novo prions exhibited higher amounts of astrogliosis and vacuolation compared to *de novo* inoculated Tg(5037) and negative control mice (Figure 3.5, 1-2). Interestingly, astrogliosis in wild type mice was diffuse and prominent throughout all brain sections (Figure 3.5A2, D2, G2). In contrast Tg(5037) mice exhibited much less reactive astrocytes in analyzed sections but more than negative controls (Figure 3.5, 2). Small amounts of dense punctate *De novo* prion deposits, characteristic of cervid prions, were identified in brain sections of Tg(5037) inoculated mice but absent in wild type inoculated mice (Figure 3.5, 3). Predominant PrP^{RES} accumulation was consistently observed in the cerebellum (Figure 3.5, B3) and the dorsal medulla (Figure 3.5, H3) in *de novo* inoculated Tg(5037) mice. Within the cerebellum of Tg(5037) mice PrP^{RES} deposits were located within the granular cells while vacuolation was prominent in the white matter of the arbor vitae, contrasting other sections where vacuolation and PrP^{RES} deposition is observable in the same area (Figure 3.5 B, 2-3). Interestingly, wild type mice and Tg(5037) mice exhibited notable neuropathologic differences of the hippocampus, notably, wild type mice did not exhibit vacuolation or PrP^{RES} staining but had appreciable amounts of reactive astrocytes while Tg(5037) had observable vacuolation, astrogliosis, and PrP^{RES} deposits in this area (Figure 3.5 D-F 1-3).


H&E (1)
GFAP (2)
PrP^RES (3)
A
B
C
D
E
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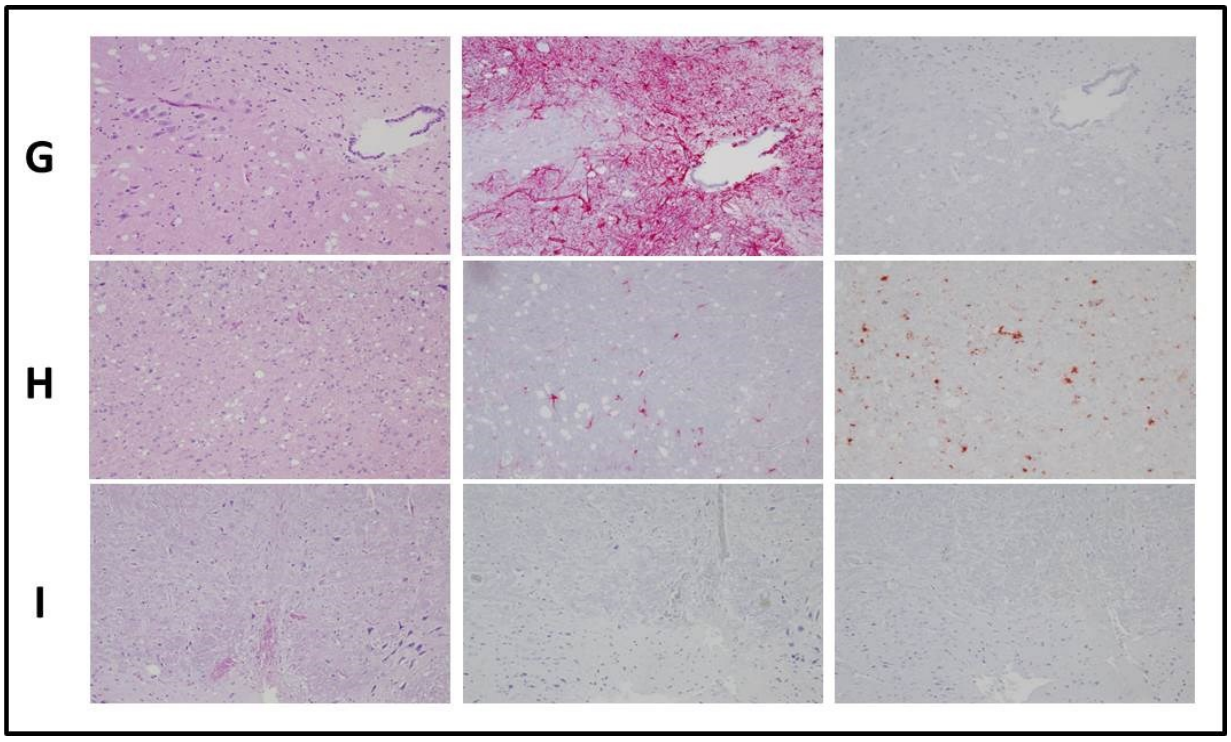

Figure 3.5. Brain sections from *de novo* inoculated terminally sick Tg(5037) **(B,E, H)**, non-clinical wild type **(A,D,G)** and uninoculated wild type **(C,F,I)** mice were assessed for vacuolation **(H&E 1)**, astrogliosis **(GFAP 2)**, and PrP^{RES} deposition **(PrP^{RES} 3)** in the cerebellum **(A-C)**, hippocampus **(D-F)** and the medulla **(G-I)**. Tg(5037) mice exhibited dense punctate PrP^{RES} plaques in all three areas whereas PrP^{RES} staining was absent from wild type and control mice **(A-I, 3)**. Diffuse GFAP staining was identified in wild type mice but was less prominent and more focal in Tg(5037) mice **(A-I, 2)**. Vacuolation was observed in wild type and Tg(5037) mice but was absent or less intense in the hippocampus **(A-I, 1)**. Negative control is mock inoculated wild type mouse >300 dpi.

We assessed neuropathology in 7 additional brain areas and generated a lesion profile averaging the total score for vacuolation, astrogliosis and PrP^{RES} deposition for all areas in *de novo* inoculated mice. Scores ranged from 0-4 with 0 correlating with no observable neuropathology to 4 correlating with severe neuropathology. Analysis of total lesion profile scores represented in radar plots (Figure 3.6, A-D) revealed that wild type mice inoculated with *de novo* prions had similar neuropathologic trends as compared to RML inoculated wild type mice, even though *de novo* inoculated mice were not clinical >500 dpi (Figure 3.6, A,D). Interestingly, *de novo* inoculated Tg(5037) mice

that were clinical and had PrP^{RES} present in most brain areas, exhibited a unique lesion profile while demonstrating less severe neuropathology compared to other strains (Figure 3.6 B). Separation of total lesion scores into categories attributed these differences to the amount of astrogliosis observed (Figure 3.6 E). Wild type mice exhibited intense GFAP staining in all brain areas and moderate vacuolation in these areas except the hippocampus. In contrast, Tg(5037) mice had little to no GFAP staining in brain areas but vacuoles were present in similar patterns compared to wild type mice (Figure 3.6E). PrP^{RES} deposition in *de novo* inoculated *Tg*(5037) mice was predominate in the reticular formation, medulla, and cerebellum contrasting D10 inoculated mice (Figure 3.6 B,D,E). Finally, a notable difference of *de novo* inoculated Tg(5037) mice was observed in the hypothalamus where neuropathology was completely absent (Figure 3.6 B,E).

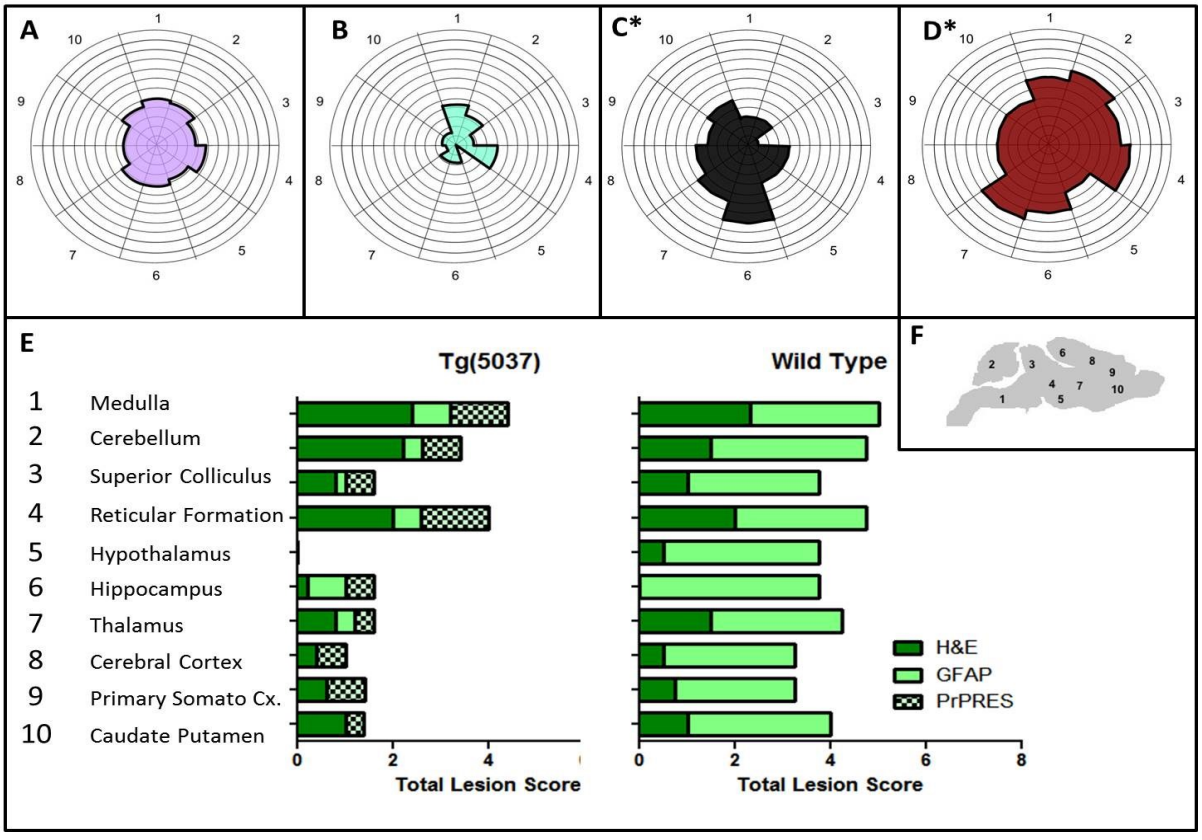

Figure 3.6. Neuropathology of 10 brain areas **(F)** were scored from 0-4 (0 indicating no neuropathology and 4 indicating severe neuropathology) for vacuolation (H&E), astrogliosis (GFAP), and PrP^{RES} deposition. Scores were plotted on radar plots for comparison **(A-D)**. *De novo* prions inoculated into wild type generated a radar plot pattern that was similar but less intense than RML prions, even though these mice were not clinical and PrP^{RES} deposition was absent in all sections **(A,D)**. In contrast, *de novo* prions inoculated into Tg(5037) mice exhibited a unique profile in lesioned brain regions and less severe neuropathology overall **(B)**. Separation of lesion scores by criteria revealed noticeable differences in GFAP staining between Tg(5037) and wild type inoculated mice **(E)** which can be attributed to vacuolation and PrP^{RES} scores **(E)**. **(F)** Anatomical diagram of brain areas analyzed. ***** Data has been previously published.

DISCUSSION

Evidence supporting spontaneous generation of prions in humans as sCJD[28,29], has led to many related hypotheses about the inception of some animal prion diseases such as chronic wasting disease and scrapie. However, proving a spontaneous event over natural transmission is challenging, especially in wild populations like cervids. Currently, the only way to attack challenging questions about biological mechanisms and the conditions needed to form *de novo* prions is through transgenic mouse models and *in vitro* assays.

Several prion research groups have reported the generation of *de novo* prions *in vitro* through recombinant technology,[30-33] PMCA[17,18] and inadvertently, by binding PrP^{C} and presumable co-factors to metal[30]. In previous reports d*e novo* generation of PrP^{RES} utilizing PMCA required addition of synthetic polyanions and/or co-purified lipids, and modification of PMCA by increasing incubation and sonication cycles[17,18]. Prion seeded PMCA experiments in our laboratory rarely generate false positives in negative controls but usually they are observed after 5 rounds of PMCA, are not reproducible in replicate samples, and contamination cannot be dismissed. In order to determine the possibility of false positives arising from spontaneous generation of prions we tested our standard

PMCA protocol in a new, prion free lab with new equipment and reagents. After 4 rounds of 48 cycles of PMCA, PK resistant material was detected by western blot in 1 of 60 total samples or at a rate of 1.6%. False positive spontaneous prions were ultimately produced at a low frequency of 6.7% after 7 rounds of standard PMCA. Therefore, we report generation of *de novo* prions at a low frequency by standard PMCA methods, using normal cervidized brain homogenate, in a prion free environment.

Barria et al., investigated the occurrence of spontaneous conversion of various specie substrates using PMCA in their laboratory[18]. They reported that they were not able to generate *de novo* prions by standard PMCA protocols and were only able to do so by extending the number of PMCA rounds and cycles where 240 cycles (5 days) constituted one round. Intriguingly, the *de* novo prions they generated were infectious and produced a new disease phenotype. We hypothesize that we generated spontaneous cervid prions without modifications and additions to our standard PMCA protocol as a consequence of the unique rigid loop in the globular domain of cervid PrP^{C}. Sigurdson et al., reported that altering the flexible loop of mouse PrP at amino acids 170 and 174, reminiscent of the rigid loop in cervid PrP, resulted in spontaneous prion disease *in vivo*[34]. Additionally, Kyle et al., reported that cell free misfolding and conversion assays of recombinant mouse PrP containing cervid amino acid substitutions for the rigid loop, had a higher misfolding propensity as compared to wild type recombinant PrP[22]. Although we have never had an occurrence of spontaneous disease in young or aged transgenic cervid overexpressors, it is plausible that the rigid loop structure of cervid PrP confers susceptibility to misfolding under pressures applied by PMCA. Transmission efficiency and prevalence of CWD prions in wild and captive

cervids[35-40] suggests that cervid PrP^C has a higher propensity for misfolding[22], although the necessary factors and conditions for increasing transmission efficiency are still unknown.

We tested infectivity of *de novo* prions in Tg(5037) cervidized mice and wild type mice resulting in clinical disease of cervidized but not wild type mice. D*e novo* inoculated cervidized mice had a similar survival to D10 inoculated cervidized mice while wild type mice were not susceptible to either strain. Importantly, Tg(5037) mice inoculated with *de novo* prions had a statistically significant delay of 104 days until average clinical disease as compared to cervidized mice inoculated with a D10 strain serially PMCA'd (sPMCA p1). sPMCA p1 was produced similarly to *de novo* through serial PMCA rounds but sPMCA was a seeded reaction. If *de novo* prions were identical to D10 we would have expected to see similar biological characteristics as other groups have reported that PMCA propagates strain characteristics[14,41,42]. In addition, we have shown that PMCA recapitulates *in vivo* strain adaptation and therefore, if *de novo* prions were identical to D10, transmission should be much more efficient as indicated by a shorter incubation period[15].Lastly, species barrier studies suggest that transmission efficiency is highly dependent on homology between host PrP^C primary sequence and PrP^{RES}[23,39,43,44]. Therefore, we expect that cervid PrP^C present in the brain homogenate substrate would only spontaneously misfold into a cervid prion. Consequently, we propose that PMCA generated *de novo* prions are a novel cervid strain.

Glycoform profiles provided further evidence that *de novo* prions are a cervid prions as *de novo* prions are mostly di-glycosylated. Curiously, *de novo* prions passaged through cervidized mice had a more equal di to mono-glycosylated ratio. It

would be interesting to investigate what phenotype would be observed after further passages into cervidized mice. Further biochemical analysis of *de novo* prions by a conformational stability assay revealed a similar profile to D10 sPMCA prions but inoculation of *de novo* prions into Tg(5037) mice stabilized the strain in contrast with sPMCA p1 prions, which were less stable. The increased stability upon passage into Tg(5037) mice may contribute to the variation in onset of disease and increased dpi[45,46].

Neuropathologic analysis of Tg(5037) mice inoculated with *de novo* prions showed PrP^{RES} deposition characteristic to CWD dense punctate plaques. However, total lesion profile scores suggested minimal neuropathology as compared to D10, RML, and even *de novo* inoculated wild type mice. *De novo* prions ultimately produced a unique neuropathologic phenotype in Tg(5037) mice as compared to other strains, again suggesting *de novo* prions are a novel cervid strain. Curiously, wild type mice exhibited more neuropathology than Tg(5037) in the absence of PrP^{RES} staining and clinical signs. Total lesion profile scores of wild type mice were highly skewed because of intense astrogliosis in all brain sections. Even in the absence of PrP^{RES} detection by western blot and IHC, wild type mice exhibited vacuolation in most areas of the brain. It is possible that old age (>500 days) can be attributed to observed neuropathology. Interestingly, these mice displayed a very similar but less severe lesion profile, when compared by radar plot, to RML inoculated wild type mice. It is possible that the wild type mice were subclinical at study termination. Castilla et al., reported that a species barrier between Tg(porcine) mice and low dose BSE prions resulted in a subclinical infection that became evident only after re-passage into Tg(porcine) mice[47]. However, the subclinical mice did not show any neuropathology as demonstrated in this study. It

is also possible that the *de novo* inoculum was a low titer that was not able to efficiently transmit into wild type mice, although western blot analysis revealed strong PK resistant bands. In the future, we plan to further test the subclinical hypothesis.

In summary, our lab generated a novel *de novo* cervid prion strain that was infectious to cervid transgenic but not wild type mice. Infected mice displayed characteristic clinical signs of prion disease including ataxia and weight loss and days to clinical onset of disease was reminiscent of a CWD prion strain. Further biochemical and neuropathologic analysis revealed a unique phenotype of *de novo* prions upon passage through Tg(5037) mice. Production of *de novo* prions *in vitro* using PMCA is a rare event that was only observed in 4 out of 60 samples after 7 rounds of standard PMCA. We feel that our data provides further evidence that cervid PrP^{C} has a higher propensity for misfolding as compared to other species under the right conditions.

REFERENCES

1. Brown, J. H. A. A. P. *et al.* Neuropathological Diagnostic Criteria for Creutzfeldt&###x2010;Jakob Disease (CJD) and Other Human Spongiform Encephalopathies (Prion Diseases). *Brain Pathology* **5,** 459-466 (1995).

2. Bendheim, P., Barry, R., DeArmond, S. & Stites, D. Antibodies to a scrapie prion protein. *Nature* (1984).

3. Dearmond, S., McKinley, M., Barry, R. & Braunfeld, M. Identification of prion amyloid filaments in scrapie-infected brain. *Cell* (1985).

4. Tempst, P., Teplow, D., Le Hood & Prusiner, S. B. A cellular gene encodes scrapie PrP 27-30 protein. *Cell* (1985).

5. Büeler, H. *et al.* Mice devoid of PrP are resistant to scrapie. *Cell* **73,** 1339-1347 (1993).

6. Hope, J., Morton, L., Farquhar, C. & Multhaup, G. The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain …. *The EMBO* (1986).

7. Prusiner, S. B. Novel proteinaceous infectious particles cause scrapie. *Science* **216,** 136-144 (1982).

8. Prusiner, S. B. Prion Diseases and the BSE Crisis. *Science* **278,** 245-251

9. Castilla, J., Saá, P., Hetz, C. & Soto, C. In Vitro Generation of Infectious Scrapie Prions. *Cell* **121,** 195-206 (2005).

10. Kurt, T., Telling, G. C., Zabel, M. & Hoover, E. Trans-species amplification of PrP CWD and correlation with rigid loop 170N. *Virology* 1-9 (2009).

11. Murayama, Y., Yoshioka, M., Yokoyama, T. & Iwamaru, Y. Efficient in vitro amplification of a mouse-adapted scrapie prion protein. *Neuroscience* (2007).

12. Thorne, L., Holder, T., Ramsay, A. & Edwards, J. In vitro amplification of ovine prions from scrapie-infected sheep from Great Britain reveals distinct patterns of propagation. *BMC veterinary* (2012).

13. Nemecek, J., Nag, N., Carlson, C. & Schneider, J. Red-Backed Vole Brain Promotes Highly Efficient In Vitro Amplification of Abnormal Prion Protein from Macaque and Human Brains Infected with Variant …. *PLoS One* (2013).

14. Castilla, J., Gonzalez-Romero, D., Saá, P. & Morales, R. Crossing the Species Barrier by PrP Sc Replication In Vitro Generates Unique Infectious Prions. *Cell* (2008).

15. Meyerett, C. *et al.* In vitro strain adaptation of CWD prions by serial protein misfolding cyclic amplification. *Virology* **382,** 267-276 (2008).

16. Green, K. M., Castilla, J., Seward, T. S. & Napier, D. Accelerated high fidelity prion amplification within and across prion species barriers. *PLoS* (2008).

17. Deleault, N., Lee, K. S., Harris, B., Caughey, B. & Rees, J. A simplified recipe for prions. in *PNAS* **104,** 9551-9552 (2007).

18. Barria, M. A., Mukherjee, A., Gonzalez-Romero, D., Morales, R. & Soto, C. De Novo Generation of Infectious Prions In Vitro Produces a New Disease Phenotype. *PLoS Pathogens* **5,** e1000421 (2009).

19. Sigurdson, C. J., Nilsson, K. P. R. & Hornemann, S. A molecular switch controls interspecies prion disease transmission in mice. *The Journal of clinical* (2010).

20. Soto, C. *et al.* De novo generation of a transmissible spongiform encephalopathy by mouse transgenesis. in *PNAS* **106,** 304-309 (2009).

21. Jiang, L., Bett, C., Eisenberg, D., Sigurdson, C. J. & Kurt, T. D. A Proposed Mechanism for the Promotion of Prion Conversion Involving a Strictly Conserved Tyrosine Residue in the 2- 2 loop of PrPC. *Journal of Biological Chemistry* 1-18 doi:10.1074/jbc.M114.549030

22. Kyle, L. M., John, T. R., Schätzl, H. M. & Lewis, R. V. Introducing a Rigid Loop Structure from Deer into Mouse Prion Protein Increases Its Propensity for Misfolding In Vitro. *PLoS One* **8,** e66715 (2013).

23. Tamgüney, G. *et al.* Chimeric elk/mouse prion proteins in transgenic mice. *Journal of General Virology* **94,** 443-452 (2013).

24. Angers, R. C. *et al.* Chronic Wasting Disease Prions in Elk Antler Velvet. *Emerging Infectious Diseases* **15,** 696-703 (2009).

25. Nichols, T. *et al.* Detection of protease-resistant cervid prion protein in water from a CWD-endemic area. *Prion* **3,** 171

26. Pulford, B., Spraker, T. & Wyckoff, A. C. Detection of PrPCWD in feces from naturally exposed Rocky Mountain elk (Cervus elaphus nelsoni) using protein misfolding cyclic amplification. *Journal of Wildlife* (2012).

27. Race, R., Raines, A., Baron, T. & Miller, M. Comparison of abnormal prion protein glycoform patterns from transmissible spongiform encephalopathy agent-infected deer, elk, sheep, and cattle. *Journal of* (2002).

28. Zerr, I. & Poser, S. Clinical diagnosis and differential diagnosis of CJD and vCJD. *APMIS* **110,** 88-98 (2002).

29. Will, R. G., Alpers, M. & Dormont, D. 12 Infectious and Sporadic Prion Diseases. *Cold Spring Harbor* (1999).

30. Edgeworth, J. A. *et al.* Spontaneous generation of mammalian prions. in *PNAS* **107,** 14402-14406 (National Acad Sciences, 2010).

31. Wang, F., Wang, X., Yuan, C. & Ma, J. Generating a prion with bacterially expressed recombinant prion protein. *Science* (2010).

32. Makarava, N., Kovacs, G. & Bocharova, O. Recombinant prion protein induces a new transmissible prion disease in wild-type animals. *Acta* (2010).

33. Zhang, Z. *et al.* De novo generation of infectious prions with bacterially expressed recombinant prion protein. *The FASEB journal* **27,** 4768-4775 (2013).

34. Sigurdson, C. J. *et al.* De novo generation of a transmissible spongiform encephalopathy by mouse transgenesis. in *PNAS* **106,** 304-309 (National Acad Sciences, 2009).

35. Williams, E. S., Williams, E. S., Williams, E. S., Young, S. & YOUNG, S. CHRONIC WASTING DISEASE OF CAPTIVE MULE DEER: A SPONGIFORM ENCEPHALOPATHY. *Journal of wildlife diseases* **16,** 89-98 (1980).

36. Williams, E. S. & Miller, M. W. Chronic wasting disease in deer and elk in North America. *Revue scientifique et technique (* (2002).

37. Williams, E. S. Chronic wasting disease. *Veterinary Pathology Online* 530-549 (2005).

38. Miller, M. W., Hobbs, N. & Tavener, S. Dynamics of prion disease transmission in mule deer. *Ecological Applications* (2006).

39. Browning, S. R. *et al.* Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *Journal of* 13345-13350 (2004).

40. Saunders, S., Bartelt-Hunt, S. & Bartz, J. Occurrence, transmission, and zoonotic potential of chronic wasting disease. *Emerg Infect Dis* (2012).

41. Joaqu *et al.* Cell free propagation of prion strains. *The EMBO* **27,** 2557-2566 (2008).

42. Jones, M., Peden, A. & Prowse, C. In vitro amplification and detection of variant Creutzfeldt–Jakob disease PrPSc. *The Journal of* (2007).

43. Telling, G. C. Transgenic mouse models and prion strains. *Prion Proteins* (2011).

44. Tamgüney, G. & Giles, K. Transmission of elk and deer prions to transgenic mice. *Journal of* (2006).

45. Safar, J. et al. Eight prion strains have PrPSc molecules with different conformations. Nature medicine (1998).

46. Kuczius, T. & Groschup, M. Differences in proteinase K resistance and neuronal deposition of abnormal prion proteins characterize bovine spongiform encephalopathy (BSE) and scrapie strains. *Molecular Medicine* (1999).

47. Castilla, J. Subclinical Bovine Spongiform Encephalopathy Infection in Transgenic Mice Expressing Porcine Prion Protein. *Journal of neuroscience* **24,** 5063-5069 (2004).

CHAPTER 4:

CERVID PRION PROTEIN PROMISCUITY ABROGATES TRANSMISISON BARRIERS

SUMMARY

Prions have a controversial history fueled by characteristics unlike other infectious microorganisms. The protein-only hypothesis suggests that prion pathogenesis is a consequence of cellular prion protein (PrP^{C}) misfolding into a β-sheet rich conformation, rendering it PK-resistant and insoluble (PrP^{RES}). How prions are able generate differing strains in the absence of nucleic acid is still an enigma. Evidence suggests that strains are enciphered by the PrP^{RES} conformation and acts as a template to convert cellular PrP^{C} into the same misfolded conformation. Transmission of prions from one host into another can often result in delayed incubation times, incomplete attack rates and sometimes complete resistance, a phenomenon defined as a species or transmission barrier. A strict species barrier has been reported between mice and cervid prions that cause CWD despite evidence that demonstrates CWD is very efficiently transmitted between cervids. We wanted to investigate the role of host factors in adaptation of cervid and mouse prions by challenging wild type mice with CWD that was previously adapted through cervidized mice. We report, for the first time, that we were able to adapt CWD through wild type mice after subsequent passage of non-clinical FVB mice inoculated with D10, a natural CWD isolate from deer, adapted through Tg(5037) mice 3 times. Additionally, we investigated the propensity of cervid prion protein to propagate mouse and cervid-adapted prion strains. We found that mice expressing cervid prion protein were much more susceptible to prion infection despite

the infectious prion being derived from a heterologous host. We conclude that cervid prion protein is promiscuous and is able to abrogate species barriers.

INTRODUCTION

Chronic wasting disease (CWD), a prion-mediated disease of cervids, is the only known transmissible spongiform encephalopathy (TSE) to infect a wild population[1,2]. There is ever-increasing biological and biochemical evidence suggesting that prion pathogenesis is caused by the conversion of the soluble normal host encoded protein (PrP^{C}) high in α-helical content, into an abnormal disease-causing isoform (PrP^{RES}) that is rich in β-sheets, insoluble, and partially protease resistant[3,4,5]. Unlike conventional microorganisms like viruses and bacteria, prions are devoid of nucleic acid[3], yet prions infect various hosts differently, suggesting the phenomenon of prion strains[6, 7,8]. Characteristic long incubation periods and incomplete attack rates, as consequence of primary passage of prion infected material between differing species, but often even within the same species, has been defined as the species or transmission barrier respectively[9,10, 7]

Conversion efficiency of infectious prions is most efficient when host and donor PrP^{C} are identical leading some researchers to believe that heterologous PrP blocks conversion, extending the days to onset of clinical disease[9,11]. Evidence also suggests that prion protein primary sequence predisposes PrP^{C} to fold a certain way but PrP^{RES} conformation enciphers biological characteristics of the strain providing a template for misfolding[12,13, 14].

Kimberlin et al., has completed extensive experimentation in mouse models to investigate strain properties. Through hamster bioassay, he suggested that natural

isolates of scrapie contained mixtures of prion strains and passage of strains through heterologous hosts may alter prion properties[15]. In addition, Kimberlin reported the propagation of new strains by serial passage through a heterologous host led to an eventual stabilization of incubation periods by 3 or 4 passages in most models[15,16] Importantly, Kimberlin showed that serial passage of prion strains through heterologous hosts could modify prion strain characteristics, selecting for a mutant prion strain and in most cases, the original phenotype could be rescued upon inoculation back into the original host. This data suggests that other host factors may play a role in prion propagation and misfolding[15,16]. Angers et al., provided additional evidence of these findings by showing the emergence of two distinct CWD strains from deer and transgenic cervidized mice. Propagation of one strain over the other was dependent on primary PrP^{C} sequence and PrP^{RES} conformation[17]. Pan et al., further demonstrated the interaction of dependence of PrP^{RES} with PrP^{C} by passaging mouse or hamster prions through transgenic mice that expressed both mouse and hamster prion protein[18]. Interestingly, mouse prions preferentially converted to mouse prions and hamster preferentially propagated hamster prions in co-expressing mice. This study also suggests that expression of heterologous PrP^{C} does not compete or inhibit replication, even when co-expressed with mouse PrP^{C}.

Many research groups have identified a species barrier phenomenon in wild type mice[19-25]. In particular, wild type mice appear resistant to CWD prions[24,25, 26,27, 28]. Curiously, adaptation of a cervid strain D10 created a new strain phenotype in Tg(1536) mice, through serial passage, that was biologically and biochemically reminiscent of mouse-adapted scrapie prions[25]. Therefore, we hypothesize that mouse host factors

may play a role in adaption in conjunction with host encoded PrP^{C}. In addition, it has been shown that shedding of PrP^{RES} and CWD transmission between cervids is highly efficient compared to other prion diseases of animals [24,29-33]. To investigate cervid susceptibility and the role host co-factors may play in strain adaptation and transmission efficiency, we challenged mice overexpressing murine PrP^{C}, wild type mice with normal PrP^{C} expression, and Tg5037xTgA20 mice that co-overexpress cervid and murine PrP^{C} with mouse and cervid-adapted prion strains and characterized them biologically and biochemically using standard methods. Remarkably, we report here for the first time, that we were able to generate a wild type mouse-adapted CWD strain that only caused clinical disease in inoculated animals after secondary passage. In addition, we successfully propagated mouse-adapted RML scrapie and D10 CWD strains back through cervidized mice but encountered a strict species barrier between cervid-adapted prions and hosts encoding murine PrP^{C}. Passage of mouse and cervid adapted strains through mice co-overexpressing mouse and cervid prion protein revealed that homologous strains were preferentially generated but cervid PrP^{C} expressing mice promiscuously converted both mouse and cervid adapted strains with varying degrees of efficiency while wild type mice were only susceptible to mouse-adapted strains. We conclude that cervid prion protein is promiscuous and therefore, abrogates the species barrier between cervid and mouse prion protein.

MATERIALS AND METHODS

Mice

FVB mice were purchased from the Jackson Laboratories (Bar Harbor, ME). TgA20, Tg(cerPrP)1536, and Tg(cerPrP)5037 were generated as previously described[24,34,35]. Tg5037xTgA20 mice were developed by breeding Tg(cerPrP)5037 mice

with TgA20 mice. F1's were checked for the presence of both mouse and cervid prion genes by PCR. F1's expressing both genes were bred and second generation mice PCR positive for cervid and mouse were used for experimentation. Western blot of animals using cervid specific antibody 9E9 (described earlier)[36] and mouse specific antibody R1[37] provided further evidence that mice were cervid and mouse co-expressers. All mice were bred and maintained at Lab Animal Resources, accredited by the Association for Assessment and Accreditation of Lab Animal Care International, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University.

Sources and preparation of prion inocula

10% brain homogenates were prepared in PMCA buffer (4mM EDTA, 150 mM NaCl in PBS). We diluted equal volumes of the inoculum 1:10 in 320 mM sucrose supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (Gibco) in PBS thirty minutes prior to intracerebral inoculations. Inoculum was made up of only one chosen animal that had an intermediate DPI compared to other infected animals within that group.

Cervid Prions

We used natural CWD isolates from brain tissue from a Colorado captive deer D10 isolate (D10) (previously described[24]}) and a captive elk (E2) as CWD prions for this study. D10 was inoculated into TgA20, Tg(1536), Tg(5037), FVB to create CWD adapted strains. Strain Nomenclature: the inoculum is listed first with corresponding passage number into host with corresponding PrP^C (i.e., D10 propagated through cervidized mice is denoted D10p1 (passaged once), D10p2 (passaged twice)). The

passage of D10 into a host with differing PrP^C is denoted by the host strain name followed by the passage number (i.e., D10p3FVBp3 indicates the D10 strain was passaged through cervidized mice 3 times and then through wild type mice 3 times) (Appendix A1.3-5).

Mouse-adapted Strains

The Rocky Mountain Lab strain of mouse-adapted scrapie prions passage 5 (RML5) was previously published{[38]. RML5 was passaged through TgA20, Tg(1536), Tg(5037), FVB, and Tg5037xTgA20 mice to create scrapie adapted strains. Strain Nomenclature: the inoculum is listed first with corresponding passage number into host with corresponding PrP^C (i.e.,RML passaged through wild type mice is denoted RML5 (passaged five times). The passage of RML into a host with differing PrP^C is denoted by the host strain name followed by the passage number (i.e., RML5/5037p2 indicates the RML strain was passaged through wild type mice 5 times and then through cervidized (5037) mice twice) (Appendix A1.3-5).

Prion inoculations and clinical scoring

Mice were anesthetized by Isofluorane inhalation. Thirty microliters of the inoculum was injected intracerebrally 3 mm deep through the coronal suture 3-5 mm lateral of the sagittal suture. Mice were monitored daily for clinical symptoms of prion disease, including tail rigidity, impaired extensor reflex, akinesia, tremors, ataxia, 15 % weight loss and paralysis. Mice with any four of these symptoms were scored terminally sick and euthanized.

PK digestion and western blotting

Samples were digested with 50 μg/ml PK (Roche) for 30 min at 37°C. The reaction was stopped by adding lithium dodecyl sulfate sample loading buffer (Invitrogen) and incubating at 95°C for 5 min. Proteins were electrophoretically separated through 12% sodium dodecyl sulfate-polyacrylamide gels (Invitrogen), and transferred to polyvinylidene difluoride membranes (Millipore). Non-specific membrane binding was blocked by incubation in 5% milk blocking solution (Bio-Rad) for 1 h. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated Bar224 anti-PrP monoclonal antibody (SPI bio) diluted 1:20,000 in Superblock (Pierce), washed 6 x 10 min in PBS with 0.2% Tween 20, and incubated for 5 min with enhanced chemiluminescent substrate (Millipore). Membranes were digitally photographed using the FujiDoc gel documentation system equipped with a cooled charge-coupled diode camera (Fuji). Densitometric analyses were performed using Quantity One software (Bio-Rad). To discriminate between mouse and cervid prion protein, western blots were run as mentioned above but incubated 1 h at room temperature with cervid specific 9E9 mAb 1:3000 followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (GE Healthcare) or with mouse specific R1 F(Ab) 1:1000 followed by horseradish peroxidase-conjugated anti-human secondary antibody (Pierce).

Histochemistry and immunohistochemistry (IHC)

Tissues were fixed in 10% paraformaldehyde, embedded in paraffin and 5-10 μm sections mounted on glass slides. For PrP staining tissue sections were deparaffinized, treated with concentrated formic acid for thirty minutes, then autoclaved at 121°C in

target retrieval solution (Dako) for 2 h, washed 2 x 7 min in 1X PBS, treated with 0.3% H_20_2 in methanol for 30 min and blocked for 1 hr with 5% BSA in PBS and mixed 1:1 in superblock (Pierce). Excess block was tapped off and sections were incubated with anti-PrP Bar 224 monoclonal antibody diluted 1:500 in block solution for 1 hour. Slides were then washed 2 x 7 min in PBS and incubated 30 min with Envision+HRP mouse secondary (DAKO). After another 2 x 7 min wash, slides were incubated for 5-7 min with AEC+Substrate-Chromagen (DAKO) and rinsed 2 x 7 min in PBS and counterstained with hematoxylin. Slides were rinsed in H2O, immersed in a 0.1% sodiumbicarbonate bluing reagent for 5 min, rinsed in tap water and coversliped with aqueous mounting medium (Richard Allan Scientific). Hematoxylin and Eosin (H&E) and glial fibrillary acidic protein (GFAP) staining was performed on a NexES automated IHC stainer (Ventana Medical systems, Inc. Tucson, AZ). Sections were stained with H&E for 4 min at room temperature. Sections were stained with rabbit polyclonal antisera against GFAP (diluted 1: 8) for 10 min at 37^{o}C followed by Biotinylated goat anti-rabbit Ig (mouse/rat adsorbed) for 8 min, then counterstained with hematoxylin for 4 min. Sections were visualized and digitally photographed using an Olympus BX60 microscope equipped with a cooled charge-coupled diode camera.

Glycoform Ratios

Western blots of 10% brain homogenates of infected mice were analyzed by densiometric analysis (Quantity One). Di, mono, and unglycosylated banding intensities were calculated as percentages of the total density of each PK treated sample. The averages of 2 replicate samples were plotted on a tri-plot based on calculated glycoform ratios.

CPCA and Conformational stability assay

We quantified the infectivity of our prion strains with the cervid prion cell assay (CPCA) as described previously[39]. Briefly, 10% brain homogenate was passed through an 18 gauge needle 15 times to break up aggregates and then passed through 21-, 23-, 26-, and 28 gauge needles respectively. Inocula was diluted to 1% in cold PBS lacking calcium and magnesium ions and coated and dried on 96 well plates.

A rabbit kidney epithelial (RK13) cell line (ATCC, Manassas VA) engineered to express deer prion protein, referred to as Deer5E9-S1, which is highly sensitive to deer CWD prions, was utilized in this assay in addition to RKM cells engineered to express murine prion protein, and RKV cells with no expression or prion protein used as a negative control. Twenty thousand cells per well were plated on top of dried inoculum in a volume of 100 ml per well. Cells were passaged three times at four day intervals at 1:10 split ratios. When cells reached confluence at the third passage, 20,000 cells per well were filtered onto Multiscreen IP 96-well 0.45-mm filter plates (Elispot plates, Millipore, Billerica, MA). Plates were dried at 50^{o} C and cells were digested for 90 min at 37^{o} C in 60 ml of lysis buffer containing 5 mg/ml proteinase K (PK) then terminated with phenylmethanesulfonylfluoride (PMSF) (2 mM). To expose the epitope of PrP27-30, cells were incubated in 120 ml 3 M guanidinium thiocyanate in 10 mM Tris-HCl (pH 8.0) for 10 min at room temperature then rinsed four times with 160 ml PBS. For immunodetection, wells were filled with 120 ml of filtered 5% superblock (Pierce, Rockford, IL) and incubated for one hr at room temperature. The solution was

removed by vacuum, and wells were incubated with 60 ml of 6H4 mAb, diluted 1: 5000 in TBST for one hr at RT or overnight at 4uC. Wells were rinsed four times with 160 ml of TBST then incubated with 60 ml AP-a-Mouse IgG (Southern Biotechnology Associates, Birmingham, AL), diluted 1: 5000 in TBST, after one hr at RT, the wells were rinsed four times with 160 ml TBST, followed by a final wash with PBS. Plates were allowed to dry completely. Visualization was done by adding 60 ml of AP conjugate substrate kit (Bio-Rad, Hercules, CA) at RT and rinsing twice with 160 ml water and allowed to completely dry. Images were scanned with a ImmunoSpot S6-V analyzer (Cellular Technology Ltd, Shaker Heights, OH), and spot numbers were determined using ImmunoSpot5 software (Cellular Technology Ltd, Shaker Heights, OH). Statistical analyses were performed using GraphPad Prism.

Cells lysates that were determined chronically infected by western blot analysis were re-plated and grown to confluency. Cells were re-plated on Elispot plates following the above protocol but with modifications. Briefly, cells were incubated for 1 hr with increasing concentrations of 0-5.5M guanidine hydrochloride in 0.5M increments. Cells were rinsed and then PK digested with 5ug/ml PK in cold cell lysis buffer for 1.5hr at 37^{o}C. Digestion was stopped by the addition of PMSF and the plate was processed identical to protocol mentioned above.

Plates were scanned with a ImmunoSpot S6-V analyzer (Cellular Technology Ltd, Shaker Heights, OH), and spot numbers were determined using ImmunoSpot5 software (Cellular Technology Ltd, Shaker Heights, OH). Conformational stability was calculated as Fapp=(observed-Native/unfolded-Native) and mean percentage of PrP^{RES} remaining ± SD were plotted as a function of GdnHCl concentration, and using fourth

order polynomial equations and nonlinear regression (GraphPad Prism) to fit denaturation curves for each prion strain.

Lesion profiling

Brain lesion profiling was performed as previously described[25] with slight modifications. Ten neuroanatomical regions were identified in coronal brain sections from at 5 mice of each group: 1-dorsal medulla, 2-cerebellum, 3- superior colliculus, 4-reticular formation, 5-hypothalamus, 6-hippocampus, 7-thalamus, 8-cerebral cortex, 9-primary somatosensory cortex and 10-caudate-putamen. A pathologist blinded to the group identification scored each region for vacuolation, astrogliosis and PrP^{RES} deposition using the following severity scale: normal (0), minimal (1), mild (2), moderate (3), severe (4). The average of the sum of the three scores constitutes the severity score for each region.

Statistical analyses

Survival curves were generated using Kaplan-Meier survival fractions (GraphPad Prism) One-way ANOVA with Tukey post-test analysis was performed using GraphPad Prism.

RESULTS

Adaptation of cervid prion strain does not abrogate wild type species barrier

To challenge the species barrier of wild type mice to cervid adapted CWD prions, we intracerebrally inoculated FVB mice with a CWD strain (D10) that was propagated through cervidized mice 3 times (D10p3). We hypothesized that the murine host factors present in transgenic mice, in addition to the cervid encoded PrP^{C}, would impact strain adaptation and therefore, cervid prions adapted through cervidized mice, would gain characteristics that would render wild type mice susceptible to cervidized mouse-

adapted CWD prions. Primary passage of D10p3 into FVB wild type mice did not abrogate the species barrier. Mice were non-clinical >380 dpi and did not have any observable PK resistant material by western blot using cervid and mouse PrP discriminatory antibodies (Figure 4.1A). In addition, despite observable vacuolation, no detectable PrP^{RES} was present by immunohistochemistry (IHC) (Figure 4.2). In contrast, Tg(5037) mice expressing cervid prion protein, had detectable PrP^{RES} by western blot (Figure 4.4B) and exhibited dense, punctate PrP^{RES} staining throughout the cerebellum, cerebral cortex, and hippocampus after inoculation with the same D10p3 strain (Figure 4.2). Further stabilization of this strain through Tg(5037) also failed to cross the species barrier into wild type mice. D10p4 inoculated FVB mice were non-clinical and PK resistant material was absent in brain homogenate (Figure 4.3) and by IHC at 400dpi (data not shown). To demonstrate that the absence of PrP^{RES} in the brains of FVB mice inoculated with D10p3 and D10p4 was not due to a delayed onset of disease, we inoculated TgA20 mice overexpressing murine prion protein with D10p4. It has been previously reported that TgA20 mice that overexpress mouse PrP^{C} progress faster to clinical prion disease than wild type mice [34]. However, here we report that TgA20 mice also failed to show signs of clinical disease and the presence of PrP^{RES} was not detected in brain homogenates or by IHC in mice >300 dpi (data not shown).

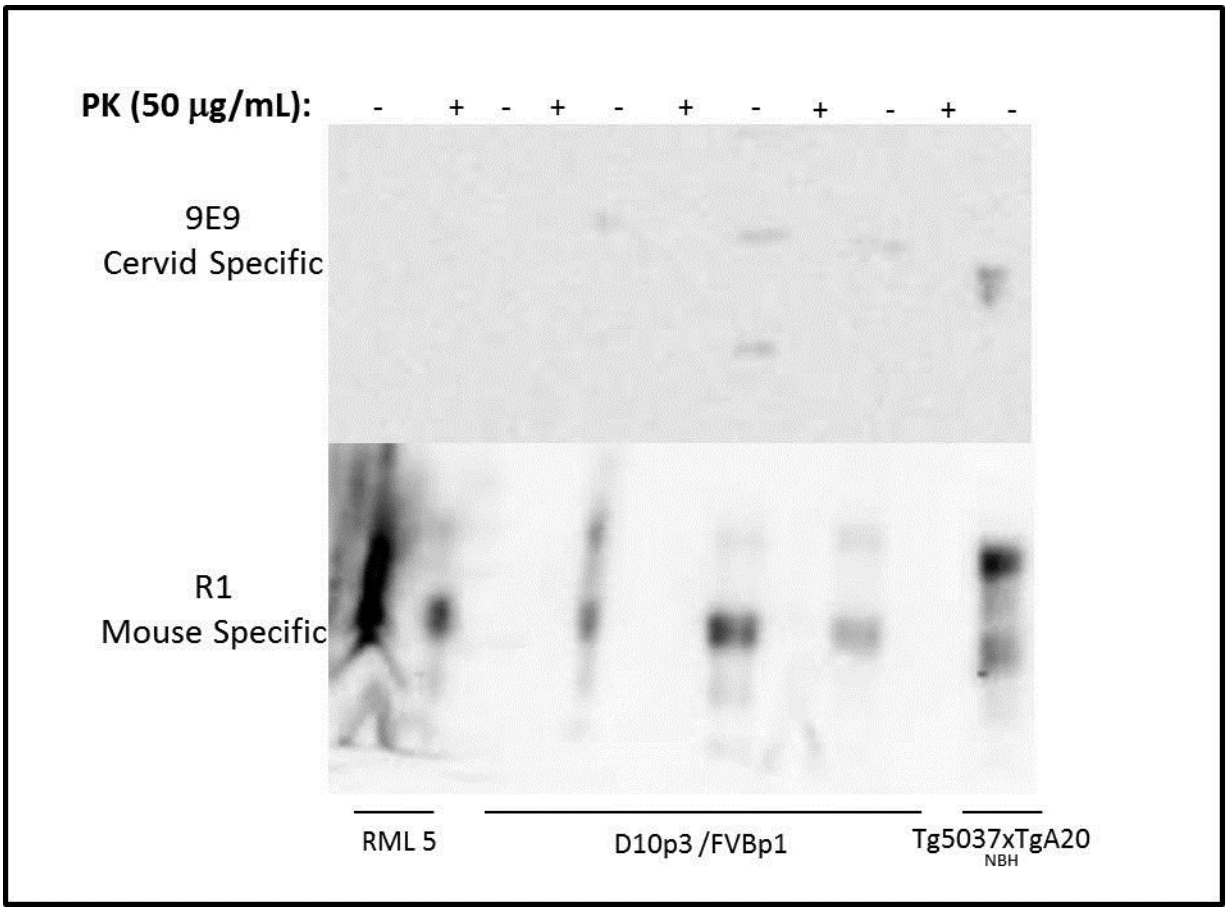

Figure 4.1. Western blot of FVB mice inoculated with CWD strain adapted through cervidized mice 3 times (D10p3/FVBp1). Western blot and antibody controls were RML 5 and Tg5037xTgA20 NBH (express mouse and cervid prion protein). Discriminatory antibodies were used to look for the presence of both mouse and cervid prions. PK resistant bands were not present in brain homogenate after >380 dpi.

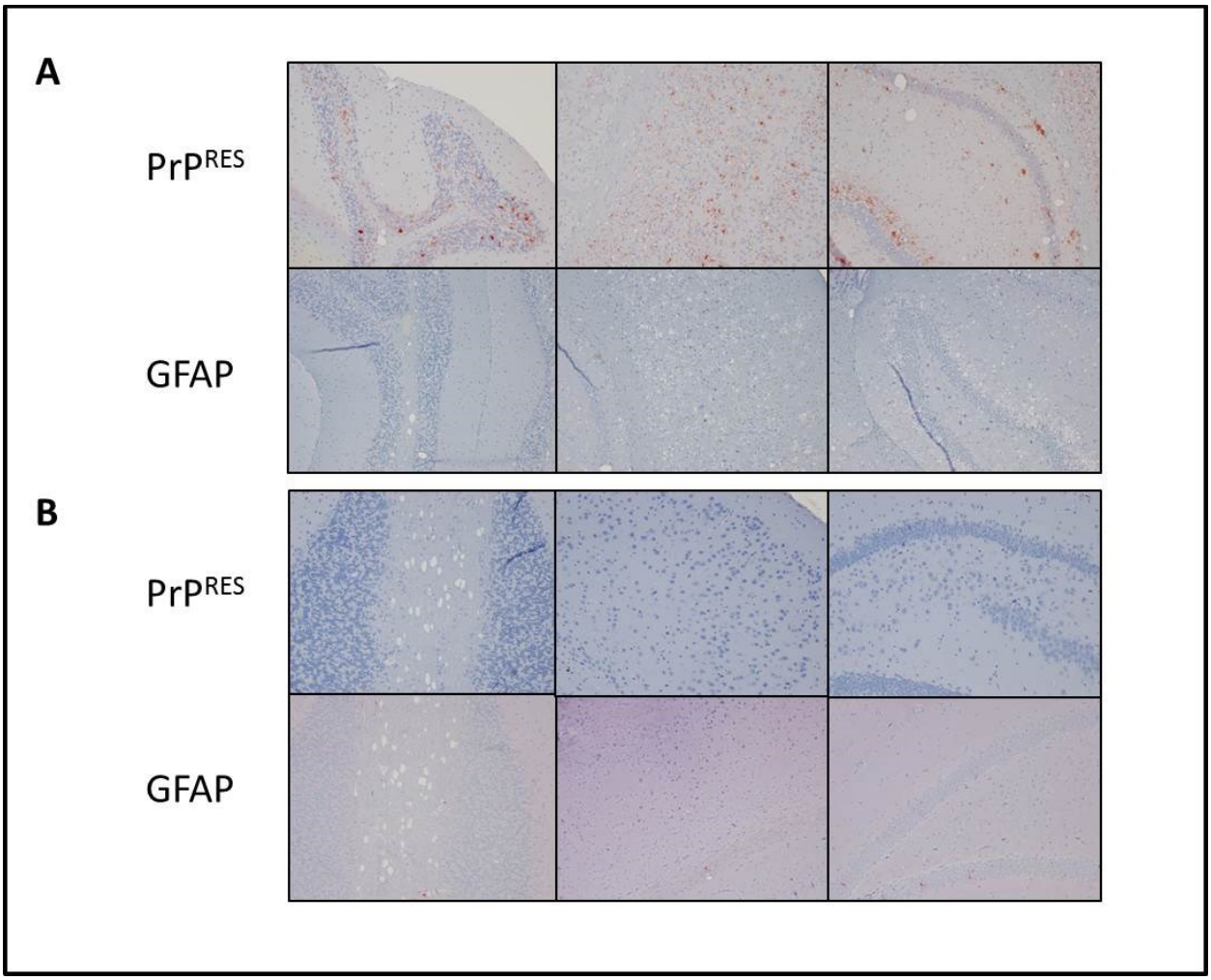

Figure 4.2. (A) Representative slides of Tg(5037) mice inoculated with same D10p3 inoculum as FVB mice in **(B)**. Tg(5037) mice developed severe vacuolation accompanied by dense PrP^{RES} deposits in the cerebellum, cerebral cortex, and hippocampus respectively. In contrast PrP^{RES} staining is absent from these areas in FVB mice. Tg(5037) and FVB mice had little to no astrogliosis present as indicated by the absence of GFAP staining.

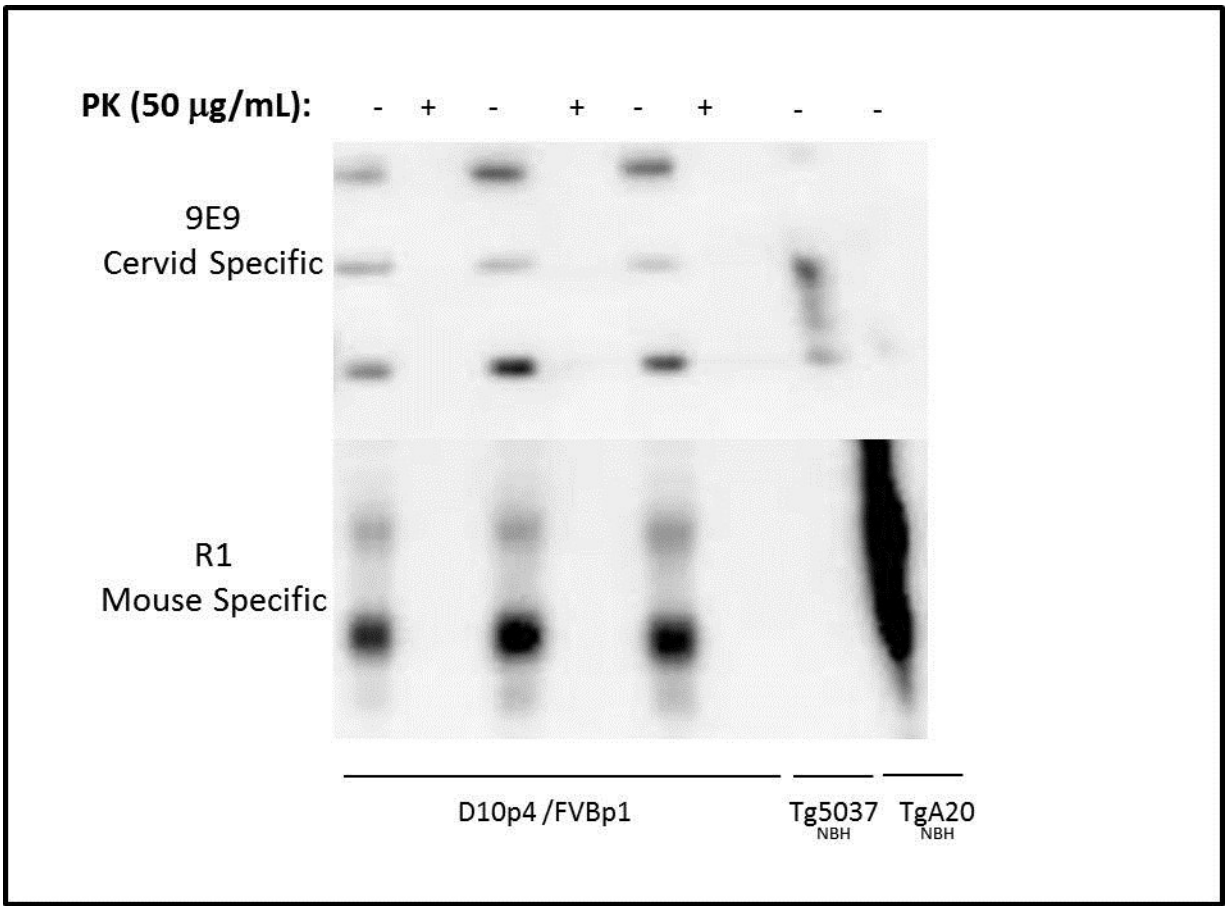

Figure 4.3. FVB mice were inoculated with brain homogenate from a cervid mouse described in Figure 4.2. D10p4 inoculation did not abrogate the wild type species barrier as observed by the absence of PK resistant material in the western blot. Tg(5037) and TgA20 normal brain homogenate were used as antibody controls.

Subsequent passage of cervid adapted prions into wild type mice abolishes the species barrier

Castilla et al., reported successful secondary transmission of BSE prions from porcine transgenic mice (poTg) inoculated with low-dose BSE[40]. First passage of the low-dose BSE inoculum into poTg mice did not cause clinical disease and PrP^{RES} material was not detected by immunoblot or IHC analysis. Surprisingly, secondary passage of the non-clinical brains back into poTg mice caused clinical disease in all animals with a greatly reduced incubation rate. To investigate the potential of a

subclinical CWD infection in wild type mice, we subsequently passaged brain homogenate from an FVB mouse that was challenged with D10p3 prions and was determined negative by clinical scoring, western blot, and IHC. Remarkably, all mice inoculated with D10p3/FVBp1 displayed signs of clinical disease in an average of 153 dpi and exhibited detectable PrP^{RES} in brain homogenate (Figure 4.4A). Interestingly, cervid and mouse discriminatory antibodies revealed that passage of D10p3 through Tg(5037) mice resulted in a cervid prion phenotype as PK resistant material was only detected with a cervid specific antibody 9E9 (Figure 4.4B). In contrast, the same inoculum passaged twice through FVB mice resulted in mouse prions (Figure 4.4A). These results prompted us to complete the same serial passage experiment of non-clinical brain homogenate from FVB mice inoculated with a natural CWD isolate, D10. Interestingly, subsequent passage of FVB brain homogenate (D10/FVBp1) back into FVB mice did not result in infection in contrast to the D10p3/FVBp1 strain.

Further passage of the newly generated mouse-adapted CWD strain into FVB mice (passage 3) further decreased the incubation period to 139 dpi. Remarkably, this strain infected FVB mice more efficiently than the mouse-adapted RML 5 strain (Table 4.1, row 2, Figure 4.5A). Additionally, we tested transmission efficiency of the newly generated mouse adapted CWD strain into TgA20 mice. Unexpectedly, inoculation of D10p3/FVBp1 (non-clinical) into TgA20 mice retarded the incubation to clinical disease as compared to RML 5 inoculation and only 4 of the 5 mice were susceptible (Table 4.1 row 5, Figure 4.5B). However, further passage into TgA20 mice (D10p3/FVBp1/TgA20p2 and p3) greatly reduced and stabilized time to clinical disease to 68 dpi. Western blot analysis using 9E9 and R1 antibodies revealed that the cervid

D10 strain was converted to mouse PrP^{RES} upon transmission into FVB and TgA20 mice.

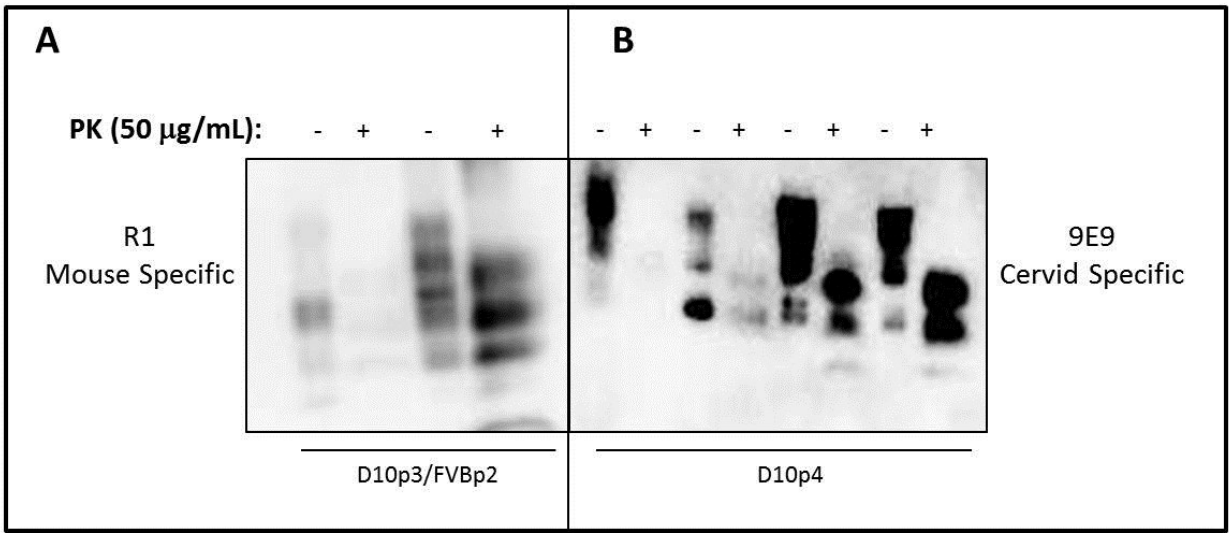

Figure 4.4. Representative western blots of infected mice. **(A)** Passage of D10p3 into FVB mice did not cause clinical disease but secondary passage of the uninfected brain homogenate (D10p3/FVBp1) into FVB mice caused clinical disease in 100% of the animals and PrP^{RES} bands were detected by western blot. The newly converted FVB prion strain was only detected with a mouse specific antibody and not cervid. **(B)** In contrast, Tg(5037) mice inoculated with the same primary strain (D10p3) was only observed when probed with a cervid specific antibody. Western blot labels indicate the resulting strain from inoculation, not the inoculum.

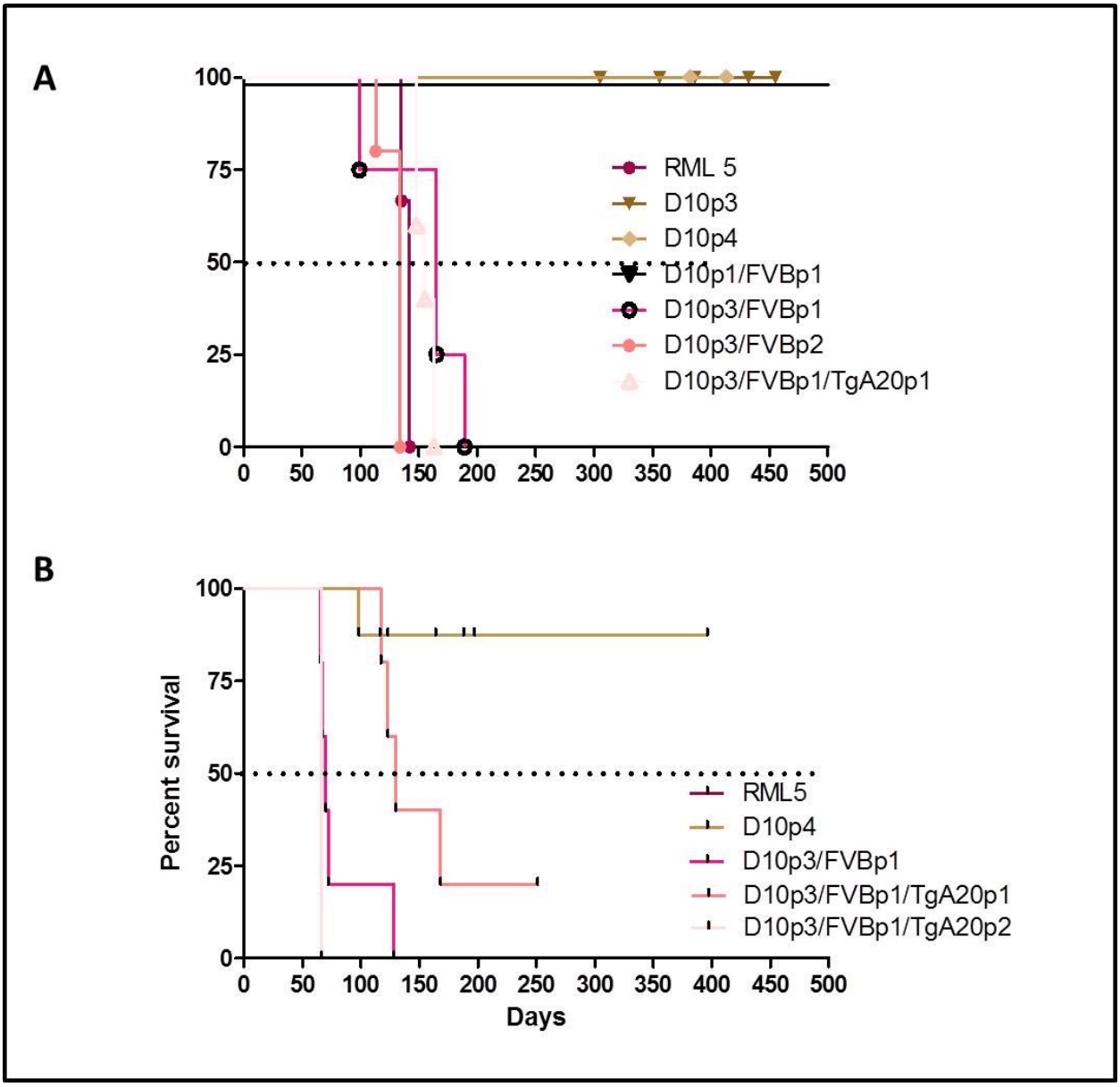

Figure 4.5. (A) Survival curves of FVB mice inoculated with a natural CWD prion strain from a deer (D10). The legend indicates the inoculum. D10 cervid prions became infectious after secondary passage into wild type mice and subsequent passages appeared to adapt the strain mimicking the survival curve of wild type mice inoculated with RML 5 mouse-adapted scrapie prions. **(B)** Survival curves of D10 inoculated TgA20 mice that over express mouse prion protein. TgA20 mice were only susceptible to D10 upon secondary passage into wild type mice. Serial passage of D10/FVBp1 into TgA20 mice adapted the strain resembling RML 5 mouse-adapted prions as seen in the wild type mice.

Cervidized mice are susceptible to FVB-adapted CWD prions but not TgA20-adapted CWD prions

To assess the biological properties of the newly generated mouse-adapted CWD prions (D10p3/FVBp2) we inoculated mouse propagated strains back into the original Tg(5037) donor. One would expect that transmission would be inefficient resulting in lengthy incubation periods and incomplete attack rates because of heterologous PrP^{C} and PrP^{RES} sequences between donor and host. However, transmission of murine CWD into Tg(5037) mice resulted in similar incubation periods of D10p2 adapted through cervidized mice and RML 5 into wild type mice with a 75% transmission rate (Table 4.1, row 6). As expected, subsequent passage of D10p3/FVBp2/Tg5037p1 into Tg(5037) mice further shortened the incubation period to 121 days and transmission was observed in 100% of the animals (Table 4.1, row 6). Curiously, mouse and cervid discriminatory antibodies revealed a sample that reproducibly reacted with the mouse specific antibody contradicting all other cervid strains and previous data showing that PrP^{RES} converted to the same isoform as the host PrP^{C} (i.e., cervid prions transmitted to mice reacted only to mouse specific antibody) (Figure 4.6). In contradiction to FVB-mouse adapted CWD data, inoculation of Tg(5037) mice with the CWD strain adapted through FVB and then throughTgA20 mice (D10p3/FVBp1/TgA20p1) prevented transmission with no detectable PrP^{RES} or clinical disease after 450 days (Table 4.1, row 8). Nevertheless, this same CWD-TgA20 strain was infectious upon passage back into FVB mice but had a delayed onset of disease as compared to TgA20 mice and FVB mice inoculated with the same parent strain before passage through TgA20 mice (Table 4.1, row 8). Importantly, TgA20 mice became resistant to the previously infectious FVB-adapted CWD strain when inoculated into Tg(5037) mice (Table 4.1, row 9).

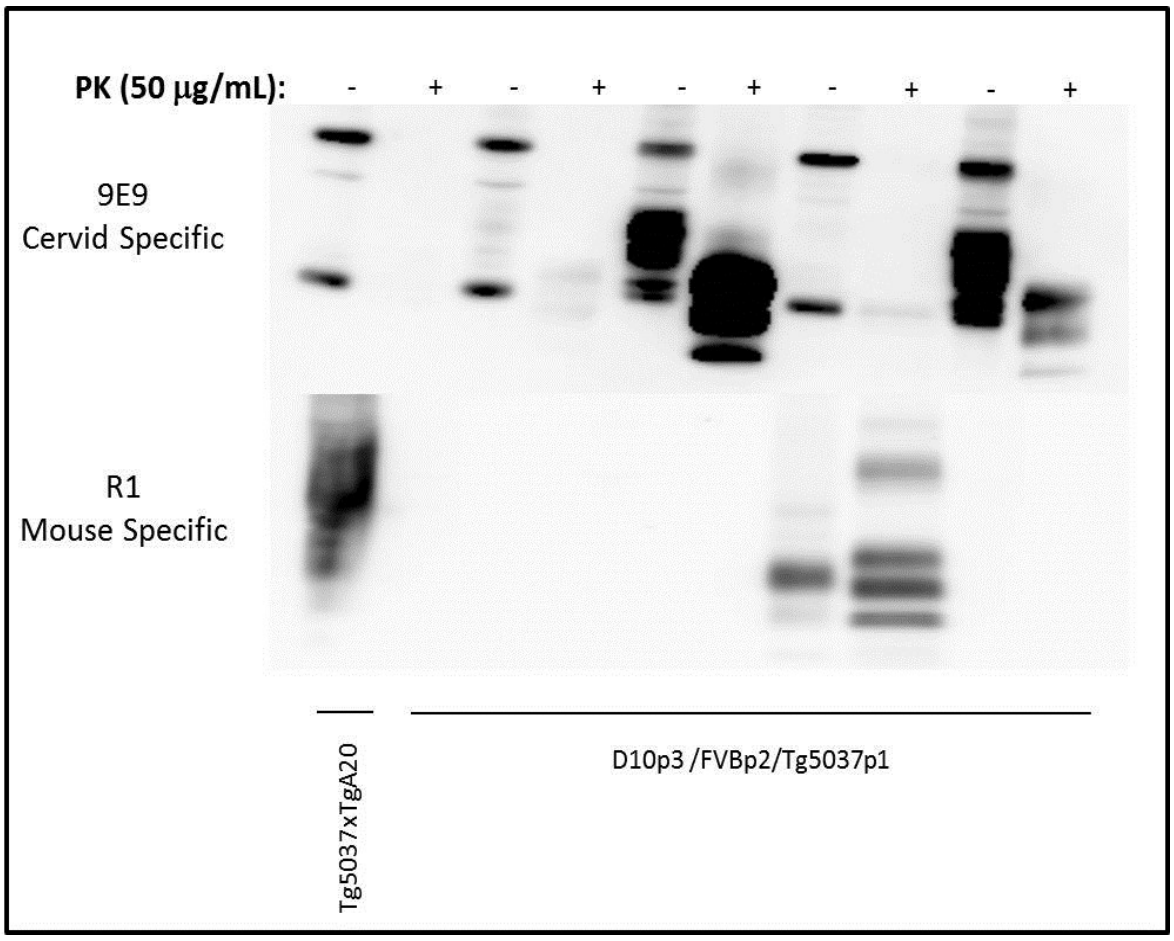

Figure 4.6. Tg(5037) mice were susceptible to FVB-adapted CWD prions. Western blot of brain homogenate from infected mice with mouse and cervid discriminatory antibodies confirmed replication of cervid prions but one cervidized mouse appeared to convert mouse prions.

Table 4.1.Table of Inoculations

[a] Mice were inoculated intracerebrally with corresponding strain. Inocula may be described in multiple rows if inoculated into multiple species i.e., D10 passage 4 for is characterized under inocula D10 and inocula D10p4.

[b] PrP^C sequence of the species in which the strain was originally derived.

[c] DPI±SD, days post inoculation to terminal disease ± standard deviation

[d] Incidence= number of terminally sick animals/number of animals inoculated

[e] PrP^c sequence of the strain after passage based on western blot using discriminatory antibodies for mouse and cervid prion protein.

	Inocula[a]	PrP^C sequence[b]	Mean DPI±SD[c] and incidence[d] →																	
			CerPrP-Tg(1536)		CerPrP-Tg(5037)					MoPrP-TgA20				MoPrP-Wild type				Mo,CerPrP-Tg5037xTgA20	
			1st passage	2nd passage	1st passage	2nd passage	3rd passage	4th passage	PrP^{RES} sequence[e]	1st passage	2nd passage	3rd passage	PrP^{RES} sequence	1st passage	2nd passage	3rd passage	PrP^{RES} sequence	1st passage	PrP^{RES} sequence
1	D10	Cervid	251±3 (8/8)	171±3 (6/6)			102±6 (5/5)	104±6 (4/4)	Cervid					>390 (0/3)					
2	D10p3	Cervid												>380 (0/5)	153±39 (4/4)	130±9 (5/5)	Murine		
3	D10p4	Cervid								>300 (0/4)				>400 (0/5)					
4	D10/FVBp1	Cervid												>500 (0/5)					
5	D10p3/FVBp1	Cervid								135±23 (4/5)	68±3 (5/5)	68±0 (5/5)	Murine						
6	D10p3/FVBp2	Cervid			168±13 (3/4)	121±12 (5/5)			Cervid										
7	D10p3/FVBp3	Cervid																83±13 (5/5)	Murine
8	D10p3/FVBp1/TgA20	Cervid			>450 (0/5)								Murine	155±7 (5/5)			Murine		
9	D10p3/FVBp2/Tg5037p1	Cervid								>485 (0/2)									
10	D10p3/FVBp3/Tg5037xTgA20	Cervid			212±122 (3/5)				Cervid	69±4 (5/5)			Murine						
11	E2	Cervid			166±18 (4/5)													265±76 (4/5)	Cervid
12	E2/Tg5037xTgA20p1	Cervid			127±135 (4/5)				Cervid	>300 (0/6)									
13	RML 5	Murine	>300 (0/6)		158±5 (3/4)	107±3 (5/5)	131±11 (5/5)		Cervid					156±8 (10/10)			Murine	97±10 (4/4)	Murine
14	RML5/Tg5037xTgA20	Murine			213±42 (5/5)				Cervid	67±2 (5/5)			Murine				Murine		
15	RML5/5037p1	Murine								80±3 (5/5)			Murine						
16	RML5/5037p2	Murine								>446 (1/3)*			Murine					216±115 (5/5)	Cervid
17	RML5/5037p2/Tg5037xTgA20	Murine			147±14 (5/5)				Cervid	>300 (0/5)									
18	Tg5037xTgA20	Cervid x Murine																>250 (0/5)	

Cervidized mice are susceptible to wild type-adapted RML prions

Browning et al., reported that Tg(1536) cervidized mice were not susceptible to RML prions after i.c. inoculation[24]. To test this conclusion in Tg(5037) mice we intracranially inoculated Tg(5037) mice with an RML prion strain that was previously adapted into CD-1 wild type mice. Unexpectedly, Tg(5037) were 75% susceptible to RML prions upon primary passage. Remarkably, days to terminal disease mimicked that of wild type inoculated with the same RML 5 strain (Table 4.1, row 13). Further passage of cervidized RML prions resulted in shortened incubation periods and complete attack rates. In contrast to FVB-adapted CWD prions propagated back through Tg(5037) mice, cervid-adapted RML prions were infectious to TgA20 mice with a 100% attack rate, although a slight delay to clinical disease was observed (Table 4.1, row 15). As expected, cervid-adapted RML was only detected by a cervid specific antibody while TgA20 mice propagated mouse specific prions (Figure 4.7).

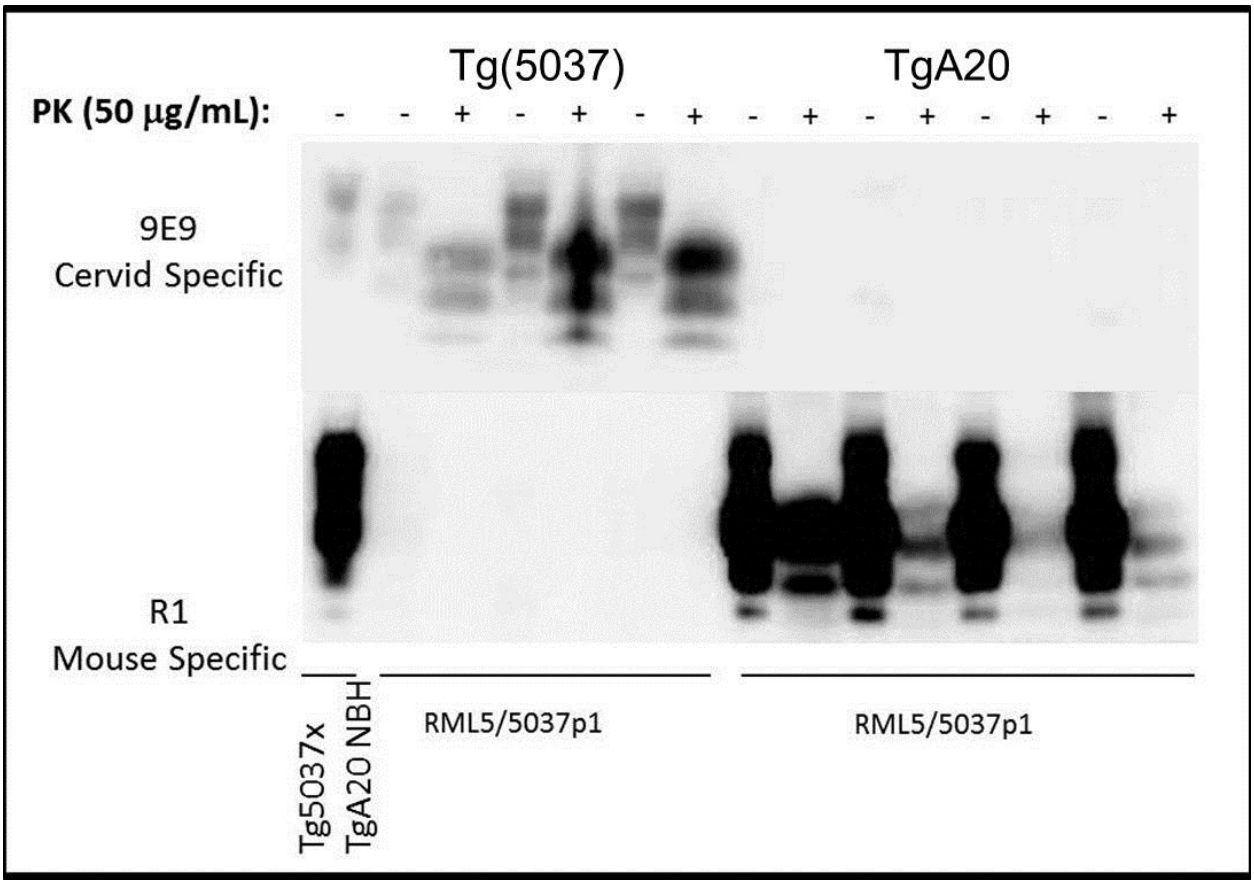

Figure 4.7. Duplicate blots demonstrate that Tg(5037) mice were susceptible to cervid-adapted RML prions and only propagated cervid prions as evident by PK resistant bands only detectable with a cervid PrP-specific antibody (top left). TgA20 mice inoculated with the same cervid-adapted RML strain were also susceptible and produced mouse prions (bottom right). Western blot labels indicate the same inoculum used for Tg(5037) mice (left) and TgA20 mice (right) and Tg5037xTgA20 NBH was a non-infected sample control for discriminatory antibodies.

Cervid and murine-adapted prion strains were infectious in transgenic mice that co-overexpressed cervid and murine PrP^C

Several research groups have investigated the prion strain phenomenon through competitive assays either by mixing strains together and inoculating or identifying two or more strains from a single inoculum and sorting out strain characteristics. Most of these experiments concluded that one strain would predominate over the other presumably by inhibiting replication or replicating more efficiently and using up available PrP^C

substrate[41-44], [15]. In addition to strain competition studies, scientists have examined the role of host PrP^C in regards to species and transmission barriers and have concluded that PrP^{RES} enciphers species specific strain properties [16,22,28]. Additionally, recent work by Tamgüney et al., demonstrated that mutations of C-terminal residues in transgenic mice expressing chimeric mouse and cervid prion protein residues, played an important role in transmission efficiency[45]. In order to test the promiscuity of cervid and mouse prion proteins to replicate various cervid and mouse CWD and scrapie strains, we generated a transgenic mouse that co-overexpressed mouse and cervid PrP^C (Tg5037xTgA20) and i.c. inoculated them with 4 different prion strains adapted through or initiating from heterologous PrP^C sequences. We hypothesized that the expression of heterologous mouse and cervid PrP^C would inhibit replication of all four strains.

Surprisingly, all of the strains caused disease but incubation times varied. Notably, inoculation of cervid strains delayed clinical disease with high variance and murine strains had a shorter incubation disease with little variance (Figure 4.8). Inoculation of D10p3/FVBp3 into co-overexpressors was the most efficient of all tested strains as mice developed clinical disease in 83 dpi (Table 4.1, row7, Figure 4.8). In contrast, E2, a CWD strain derived from a natural elk isolate, had the longest incubation period of 265 dpi and only infected 75% of the animals (Table 4.1, row 11, Figure 4.8). RML 5 caused disease in all animals within 93 dpi which is delayed compared to RML inoculated TgA20 mice but greatly reduced as compared to wild type and Tg(5037) mice (Table 4.1, row 13, Figure 4.8). Finally, inoculation of the RML5/5037p2 strain caused disease in all animals by 216 dpi (Table 4.1, row 16, Figure 4.8). Interestingly, western blot analysis of infected Tg5037xTgA20 brain homogenates with mouse and

cervid discriminatory antibodies demonstrated that the nascent PrP^{RES} sequence coincided with the last animal the strain was propagated through and not the original host's PrP^{C} sequence. Therefore, even though D10p3/FVBp3 initiated as a cervid prion strain, adaptation through wild type mice generated a mouse prion strain that preferentially converted mouse PrP^{C} in Tg5037xTgA20 co-overexpressing mice (Table 4.1. rows 7, 11, 13, and 16, Figure 4.9). Similarly, RML 5 prions reproduced mouse prions after passage through Tg5037xTgA20 mice, while subsequent passage of RML5 through Tg(5037) and then Tg5037xTgA20 mice preferentially replicated cervid prions (Figure4.9).

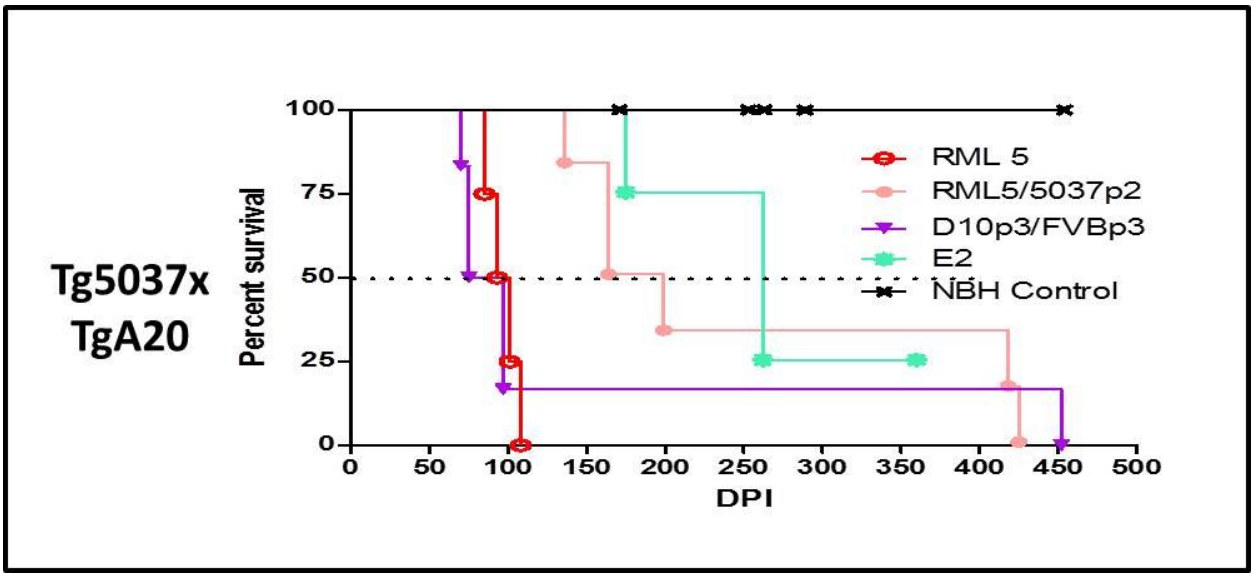

Figure 4.8. Survival curves of Tg5037xTgA20 mice that co-overexpress mouse and cervid prion protein. Inoculation of E2 (a natural elk CWD strain), RML 5 (mouse-adapted scrapie strain), D10p3/FVBp3 (mouse-adapted CWD strain), and RML5/5037p2 (cervid-adapted scrapie strain), resulted in clinical disease with varying incubation periods. All of the inoculated strains except E2 had complete attack rates.

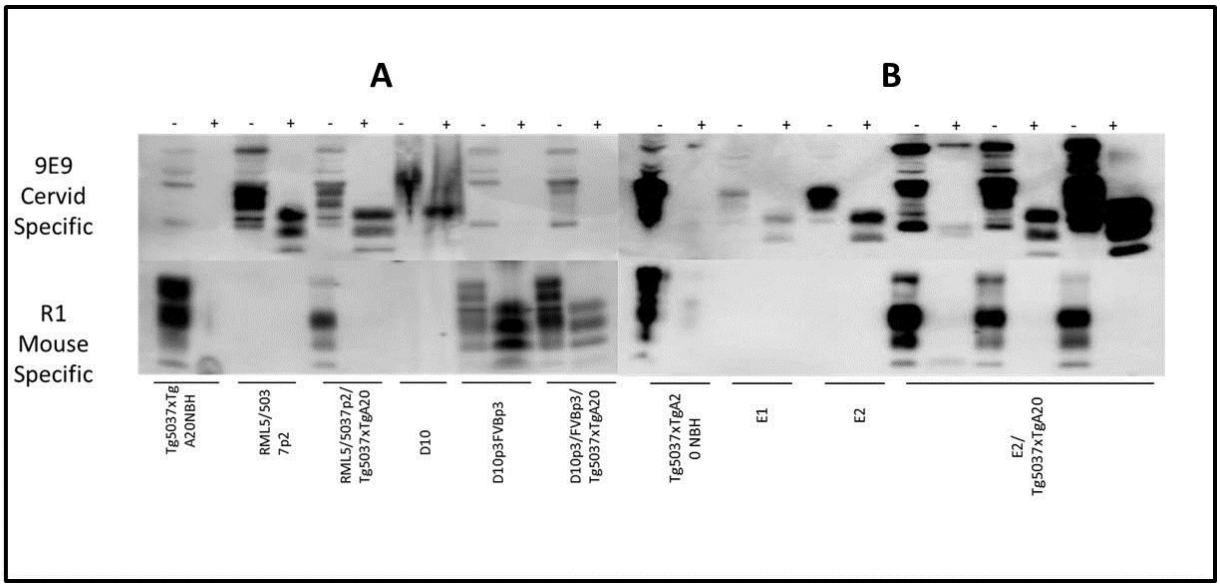

Figure 4.9. (A) Western blots probed with cervid specific 9E9 or mouse specific R1 prion antibody. Passage of RML 5 mouse-adapted prions into Tg(5037) mice generates cervid but not mouse prions and subsequent passage into Tg5037xTgA20 mice overexpressing both mouth and cervid prion protein, preferentially replicates cervid prions. Similarly, D10, a natural cervid CWD strain converts mouse PrP^C upon inoculation into FVB mice. Passage into Tg5037xTgA20 mice reproduces mouse prions from the original cervid strain. **(B)** Another natural CWD strain from elk preferentially replicates cervid prion protein after inoculation into Tg5037xTgA20 mice. The first lanes of each western blot include negative brain homogenate from uninfected Tg5037xTgA20 mice.

Inoculation of original host with strains from inoculated co-overexpressing mice suggest cervid prion protein promiscuity abrogates species barriers

To identify the presence of species barriers between newly generated strains from co-overexpressing mice and mice that express cervid or mouse PrP^C, we inoculated each of the 4 previously described strains back into the original host. As expected, cervidized mice were completely susceptible to the RML5/5037p2/Tg5037xTgA20 strain succumbing to clinical disease in 147 dpi and the E2/Tg5037xTgA20 strain, showing clinical signs in 127 dpi. Curiously, the E2/Tg5037xTgA20 strain mimicked cervid adapted CWD with a quicker disease course as compared to E2 primary passage into Tg(5037) mice and primary inoculation of E2

into Tg5037xTgA20 mice (Table 4.1, Figure 4.10A). In contrast, Tg(5037) mice exhibited a lengthened incubation of 213 dpi upon RML5/Tg5037xTgA20 inoculation. Remarkably, Tg(5037) mice showed varying susceptibility to all 4 strains replicated through Tg5037xTgA20 mice (Figure 4.10). The biggest variance was seen upon inoculation of the D10p3/FVBp3/Tg5037xTgA20 strain where only 3 out of 5 mice became infected. Passage of mouse adapted CWD (D10p3/FVBp3) and mouse-adapted scrapie (RML 5) through Tg5037xTgA20 mice did not inhibit efficient transmission in TgA20 mice yet a strict species barrier was evident after inoculation of E2/Tg5037xTgA20 and RML5/5037p2/Tg5037xTgA20 prions into TgA20 mice (Figure 4.11).

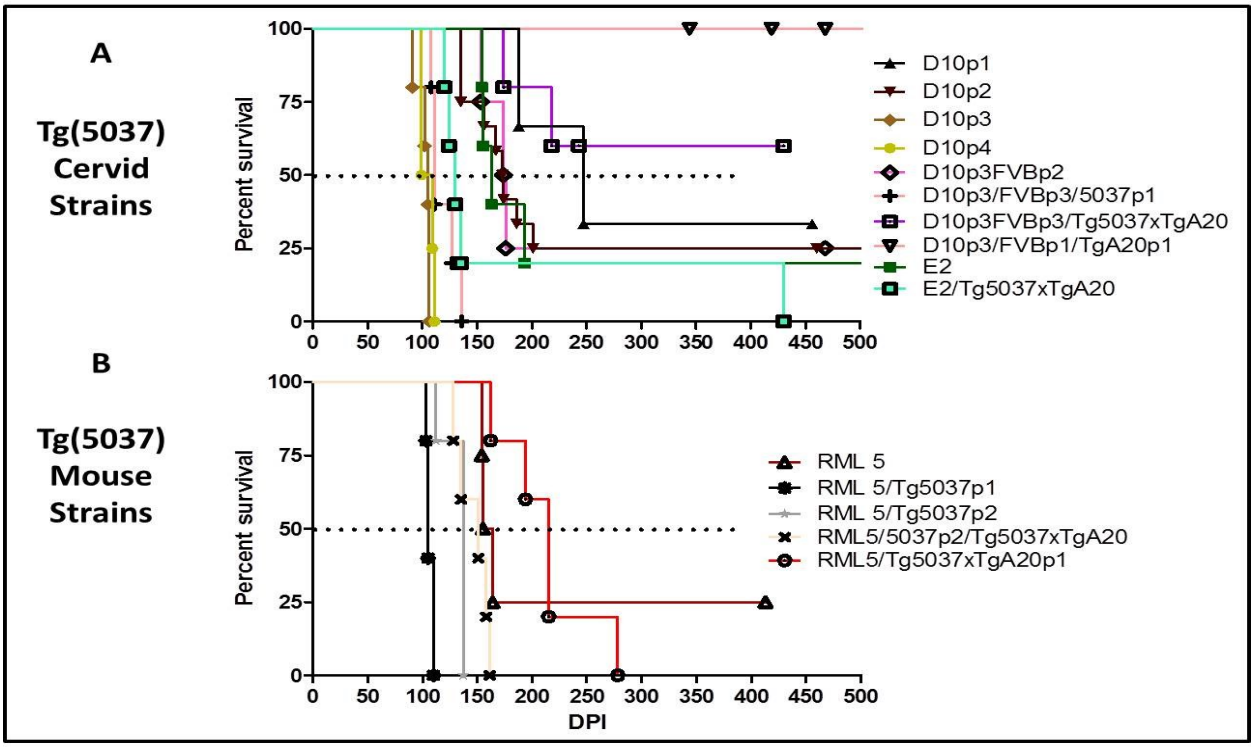

Figure 4.10. Survival curves of Tg(5037) mice inoculated with strains that began as cervid prion strains **(A)** or with strains that began as murine prion strains **(B)**. Little to no resistance to transmission was observed from strains inoculated into Tg(5037) mice except for strains previously propagated through TgA20 mice overexpressing mouse prion protein.

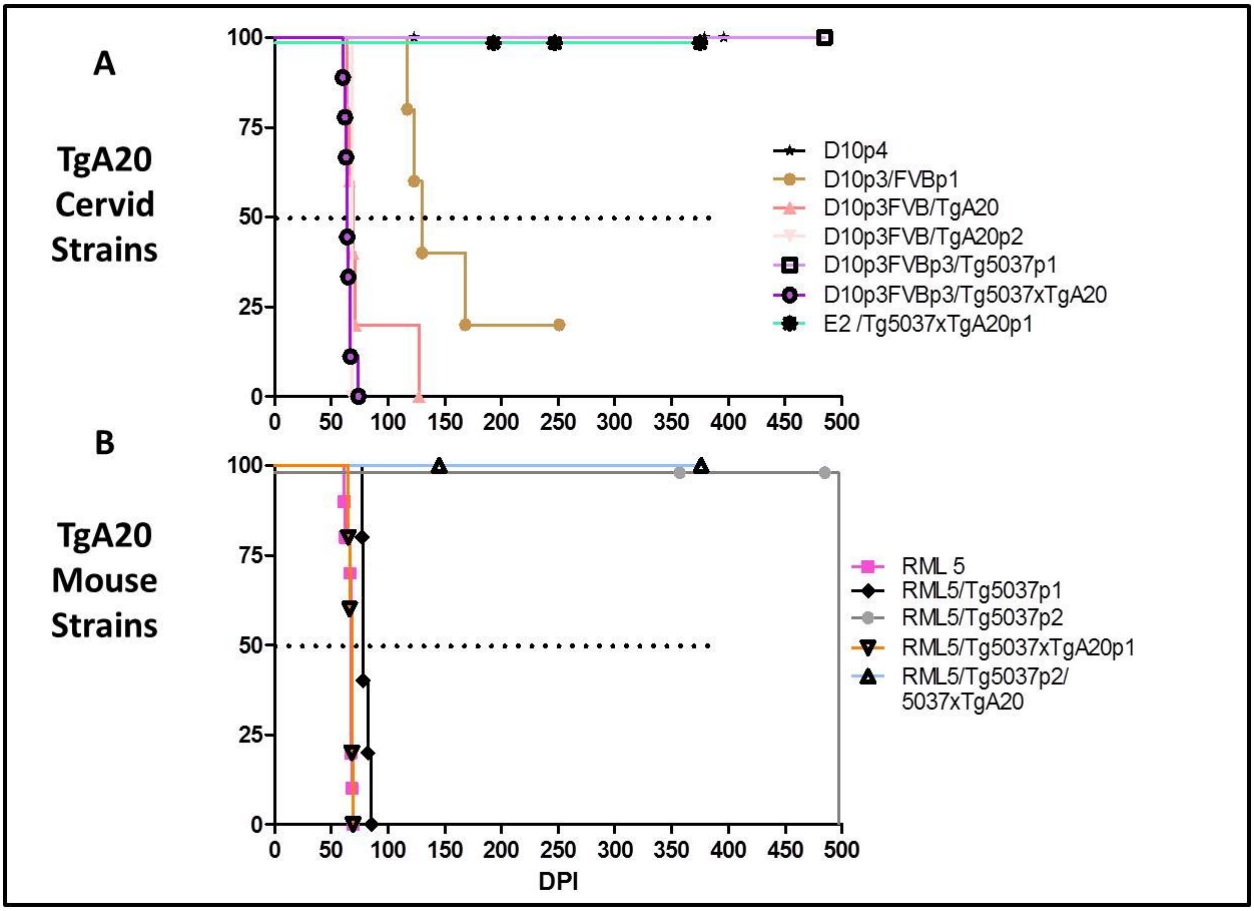

Figure 4.11. Survival curves of TgA20 mice inoculated with strains originating as cervid prions **(A)** or mouse strains **(B)**. TgA20 mice were only able to propagate murine-adapted strains once inoculated through Tg5037xTgA20 mice that co-overexpress cervid and mouse prion protein.

Cervidized mice have increased neuropathology

To compare neuropathology between cervid and mouse adapted prion strains, we scored 10 brain sections (Table 4.2) for vacuolation, astrogliosis, and PrP^{RES} deposition. Severity of neuropathology increased in Tg(5037) mice despite heterologous PrP^{C} sequences (Figure 4.12 and 4.13). Most notably, passage of RML 5 through Tg5037xTgA20 mice did not cause severe neurodegeneration as compared to cervid strains propagated through this same strain of mice (Figure 4.12A) However,

subsequent passage of RML5/Tg5037xTgA20 back into cervidized mice exacerbated disease compared to primary inoculation of RML 5 into Tg(5037) mice. Lesion profiles plotted on radar plots demonstrate that all strains propagated in Tg(5037) mice had similar neuropathologic patterns but with varying severity (Figure 4.12, Appendix A1.1). Additionally, mouse-adapted CWD (D10p3/FVBp3) propagated in Tg5037xTgA20 mice produced a similar but less intense radar plot as compared to cervid-adapted strains (Figure 4.13).

Table 4.2. Neuroanatomical areas analyzed for lesion profiling.

Lesion number	Lesion area
1	Dorsal medulla
2	cerebellum
3	Superior colliculus
4	Reticular formation
5	Hypothalamus
6	Hippocampus
7	Thalamus
8	Cerebral cortex
9	Primary somatosensory cortex
10	Cuadate-putamen

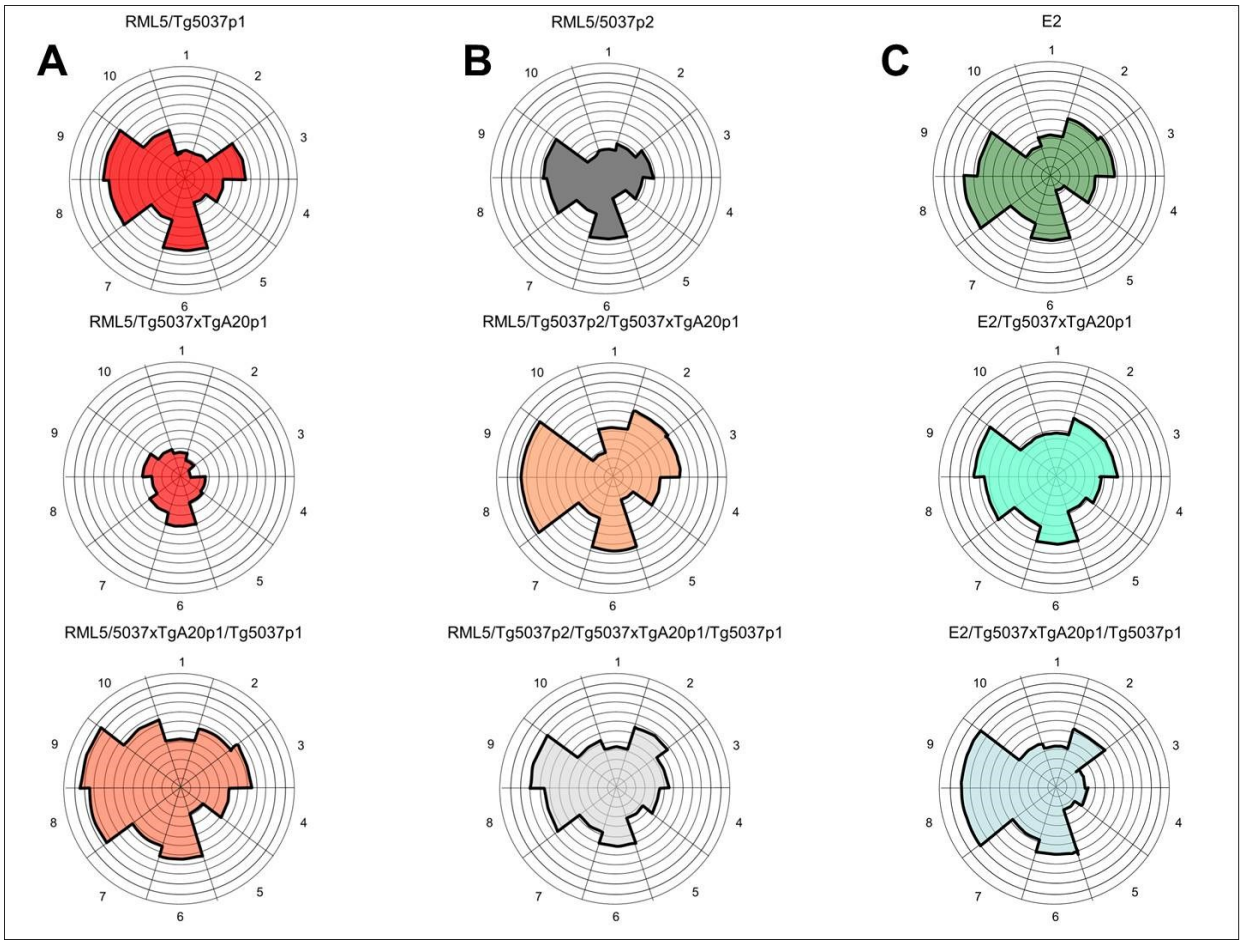

Figure 4.12. Neuropathology of terminally sick mice inoculated with mouse and cervid adapted strains were scored in 10 brain areas by presence of vacuolation, astrogliosis, and PrP^{RES} deposition. Average scores were plotted on radar plots according to neuroanatomical sections affected. RML 5 **(A)**, RML5/5037p2 **(B)**, and E2 **(C)** progression are plotted as strains are primarily propogated through Tg(5037), secondarily through Tg5037xTgA20 mice and then back into Tg(5037) mice. Tg(5037) demonstrate efficient propagation of all three strains. Unfortunately, PrP^{RES} staining was difficult to detect within FVB and TgA20 mice and within Tg(5037) mice inoculated with D10p3/FVBp3/Tg5037xTgA20 prions. Labels above individual radar plot correlate with the strain. Numbers around the radar plot correlate with associated areas analyzed for lesion profiles (Table 4.2).

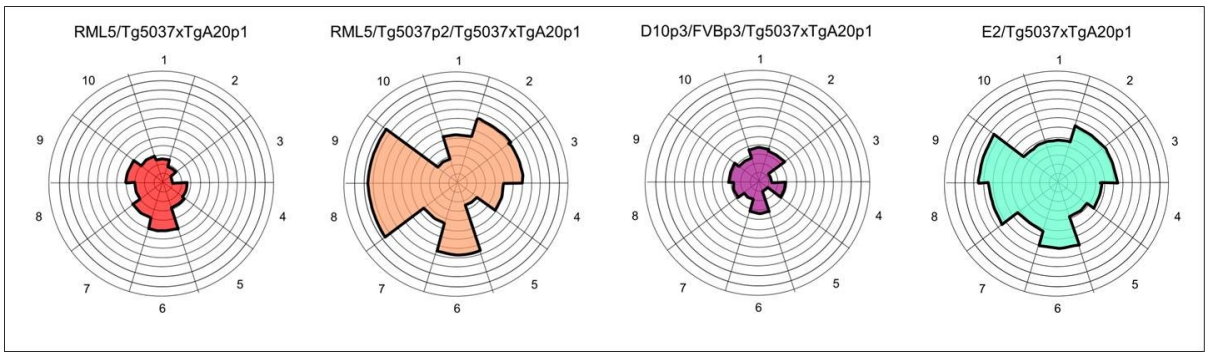

Figure 4.13. Lesion profiles of Tg5037xTgA20 mice demonstrate that cervid-adapted strains caused more severe neuropathology compared to mouse-adapted prion strains. Labels above each individual plot represent the prion strain after inoculation and the numbers around the radar plots correspond to Table 4.2. Interestingly, all three strains that have been propagated through Tg(5037) mice have similar lesion profiles despite variability in severity.

Intriguingly, PrP^{RES} detection by IHC in FVB and TgA20 mice that had been inoculated with adapted strains was minimal despite strong PK resistant material detectable by western blot. This was also observed in Tg(5037) mice inoculated with mouse-adapted strain D10p3/FVBp3/Tg5037xTgA20 (Figure 4.14). Notably, radar plots of Tg5037xTgA20 mice inoculated with either D10p3/FVBp3 or RML 5 had less severe scoring as compared to cervid-adapted strains (Figure 4.13). Even more compelling is the observation that Tg5037xTgA20 control mice that were homozygous for cervid prion protein had detectable PrP^{RES} deposition by IHC correlating with increased severity of lesion profiles as compared to Tg5037xTgA20 control mice that were homozygous for murine prion protein (Appendix A1.2). In addition, Tg5037xTgA20 mice inoculated with mouse-adapted prions had little observable vacuolation n comparison to cervid-adapted strains yet exhibited increased amounts of reactive astrocytes comparable to cervid-adapted strains (Figure 4.14 and 4.15). Tg5037xTgA20 mice had severe neuronal staining most notable in the cerebellum, cerebral cortex, and hippocampus (Figure 4.15). Dense, punctate PrP^{RES} plaques were observed characteristic of CWD prions but

diffuse staining characteristic of mouse-adapted RML was also present in brain of terminally sick Tg5037xTgA20 mice (Figure 4.15). Intense GFAP staining, a marker for reactive astrocytes, correlated in these areas of dense PrP^{RES} plaques. Furthermore, subsequent passage of mouse and cervid-adapted prions back into Tg(5037) mice efficiently propagated prions throughout the brains of infected mice (Figure 4.16). These mice also exhibited diffuse and punctate PrP^{RES} staining.

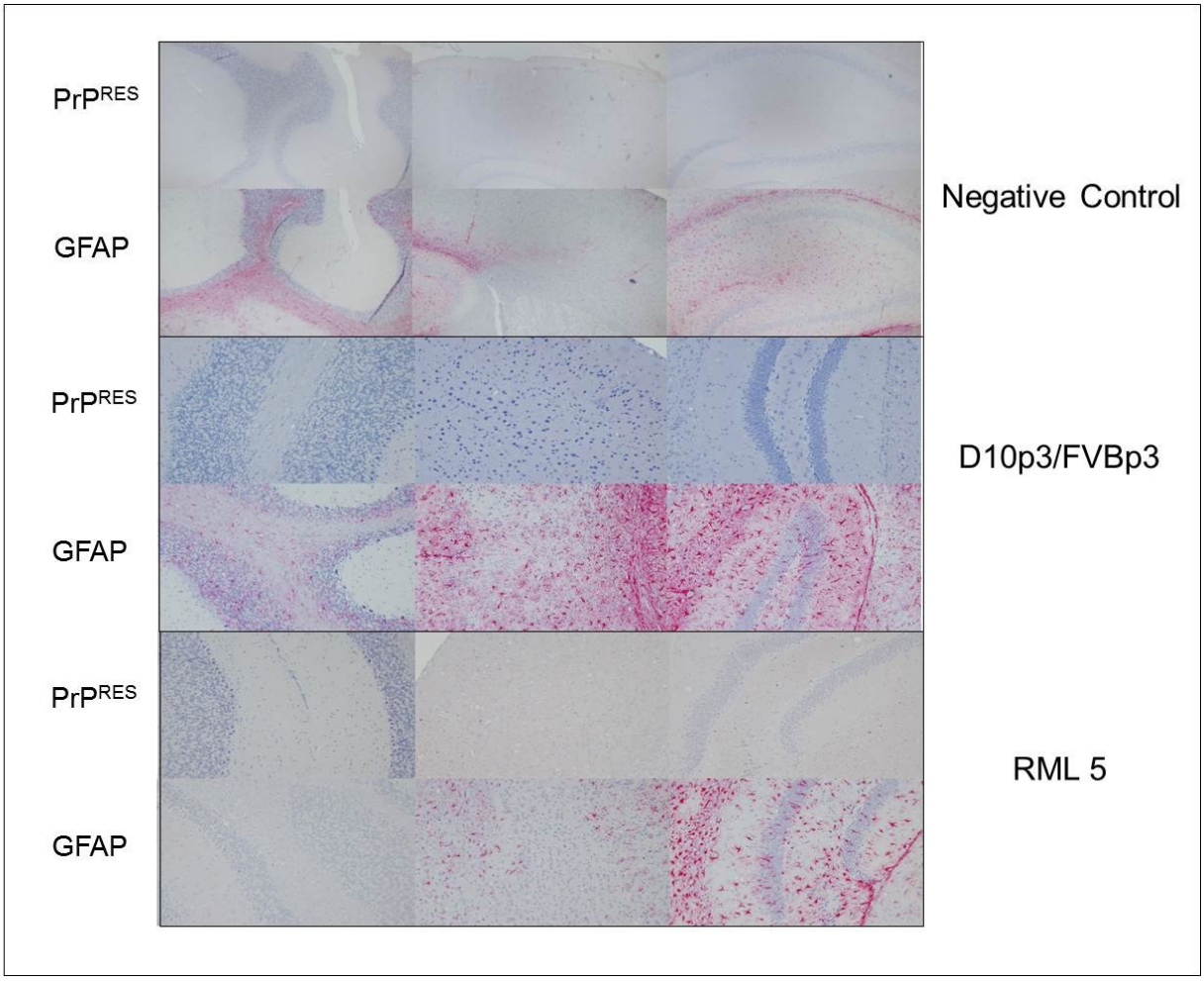

Figure 4.14. Immunohistochemistry (IHC) of terminally sick Tg5037xTgA20 mice inoculated with mouse-adapted CWD (D10p3/FVBp3) and mouse-adapted RML 5. The negative control is a mock inoculated Tg5037xTgA20 mouse. Representative sections of cerebellum, cerebral cortex, and hippocampus, respectively, demonstrate minimal to

no PrP^{RES} deposition despite significant GFAP staining signifying reactive astrocytes.

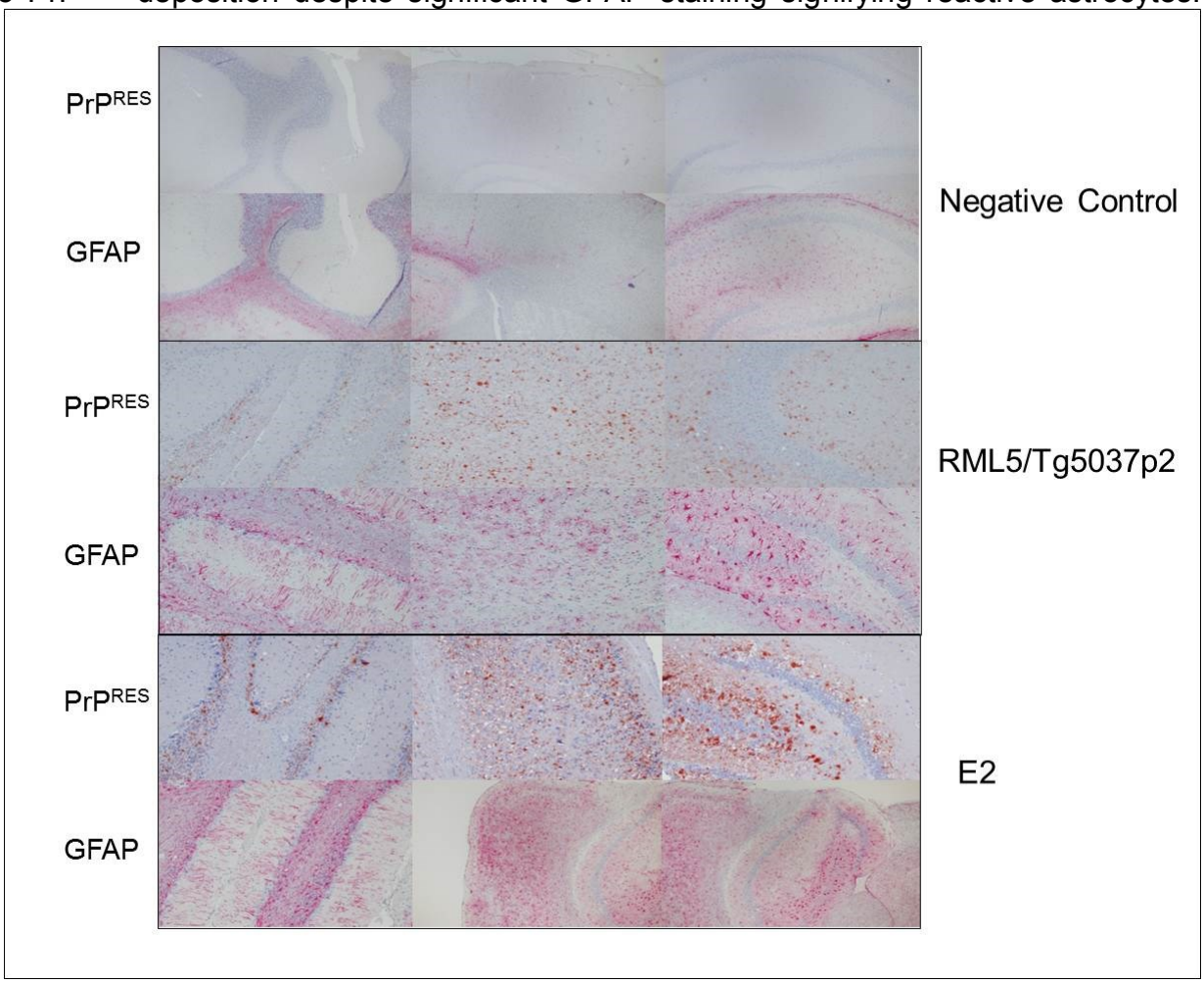

Figure 4.15. Immunohistochemistry (IHC) of terminally sick Tg5037xTgA20 mice inoculated with cervid-adapted RML 5 (D10p3/FVBp3) and primary passage of CWD natural isolate, E2. The negative control is a mock inoculated Tg5037xTgA20 mouse. Severe PrP^{RES} staining is significant in the cerebellum, cerebral cortex, and hippocampus, respectively. PrP^{RES} deposits are characteristic of both CWD and RML 5 prions. Astrogliosis is evident by intense staining for GFAP.

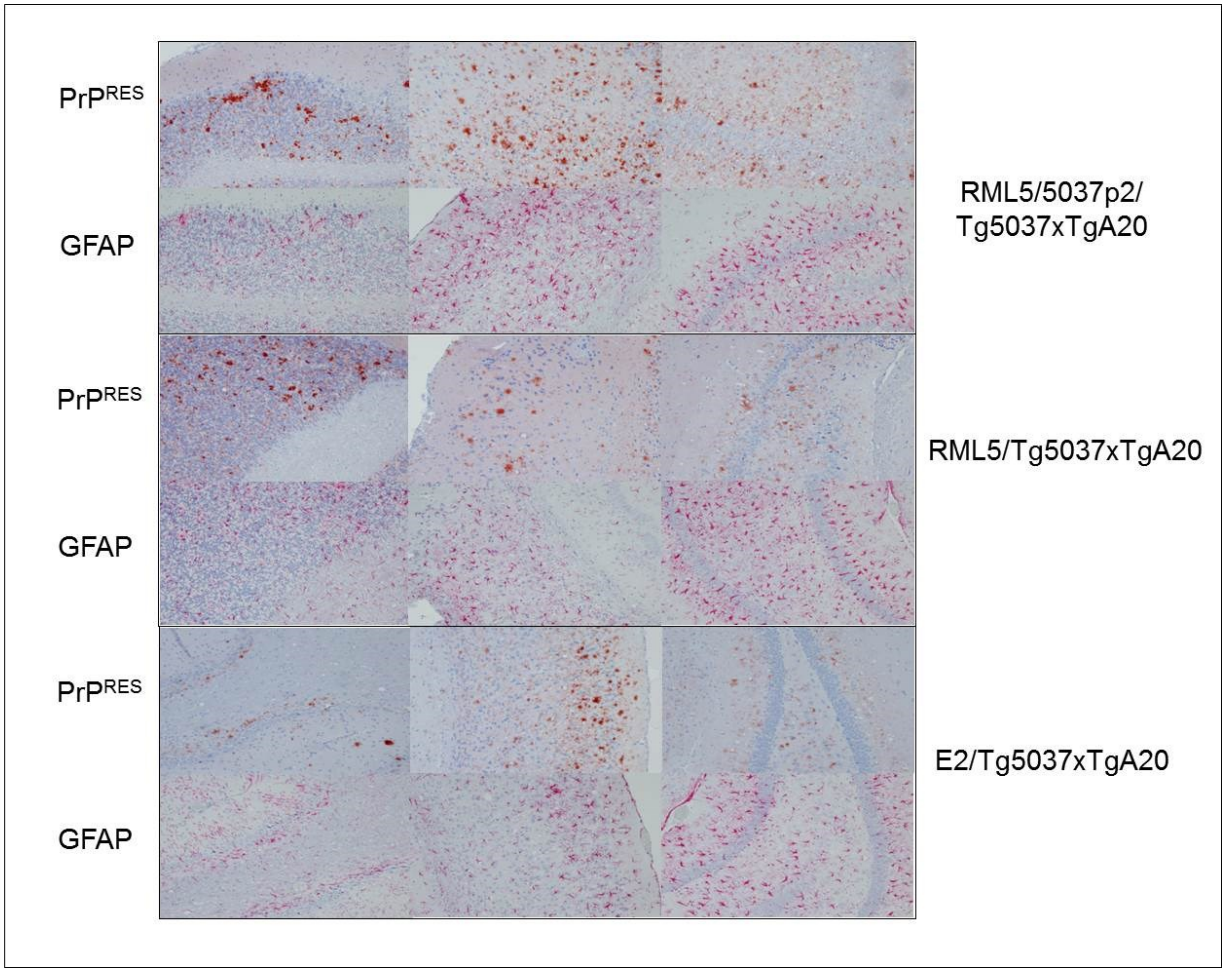

Figure 4.16. . Immunohistochemistry (IHC) of terminally sick Tg(5037) mice inoculated with strains first propagated through Tg5037xTgA20 mice. The staining is consistent with brains of Tg5037xTgA20. Dense, diffuse and punctate PrP^{RES} deposition is detectable in the cerebellum, cerebral cortex, and hippocampus respectively. Interestingly, terminally sick Tg(5037) mice inoculated with the D10p3/FVBp3/Tg5037xTgA20 strain did not have any detectable PrP^{RES} staining in any of the brain sections analyzed.

Cervid-adapted prions exhibit fickle glycoform profiles

We further assessed characteristics of newly generated mouse and cervid-adapted prions through analysis of glycoform ratio profiles. TgA20 and FVB mice propagated all prion strain with similar glycoform profiles of a higher mono-glycosylated to di-glycosylated to un-glycosylated ratio (Figure 4.17B,C). Intriguingly, TgA20 mice inoculated with RML5/Tg5037p1 prions replicated glycoform ratios identical to mouse-

adapted prions. Unexpectedly, one TgA20 mouse inoculated with RML5/5037p2 had detectable PK resistant material by western blot that was only detected by a cervid specific antibody and was highly glycosylated compared to other strains inoculated into TgA20 mice (Figure 4.17B). FVB mice remained faithful at propagated higher mono-glycosylated prions with little difference exhibited upon further adaptation of passaged strains (Figure 4.17C). Analysis of mouse-adapted CWD and cervid-adapted RML prions propagated through Tg(5037) mice revealed variable glycoform ratios that trended toward an equal ratio of di to mono-glycosylation (Figure 4.17A). Our data suggests that primary passage of a heterologous strain into Tg(5037) mice generates glycoform ratios characteristic of CWD with a high di to mono-glycosylated ratio. However, upon further passage into a mouse expressing cervid PrP^{C}, the ratio decreases (Figure 4.17A). This is evident when analyzing propagation characteristics of the mouse-adapted CWD strain. Primary passage of the D10p3/FVBp3 strain back into Tg(5037) mice produces a mostly di-glycosylated form yet subsequent passage of this strain generated a more equal distribution of glycosylation (Figure 4.17). Finally, glycoform ratios of strains propagated through Tg5037xTgA20 mice was consistent with antibody discrimination data. Mouse-adapted strains produced glycoform ratios characteristic to mouse or scrapie prions and cervid-adapted prion strains generated glycoform ratios consistent with cervid or CWD prions (Figure 4.17D).

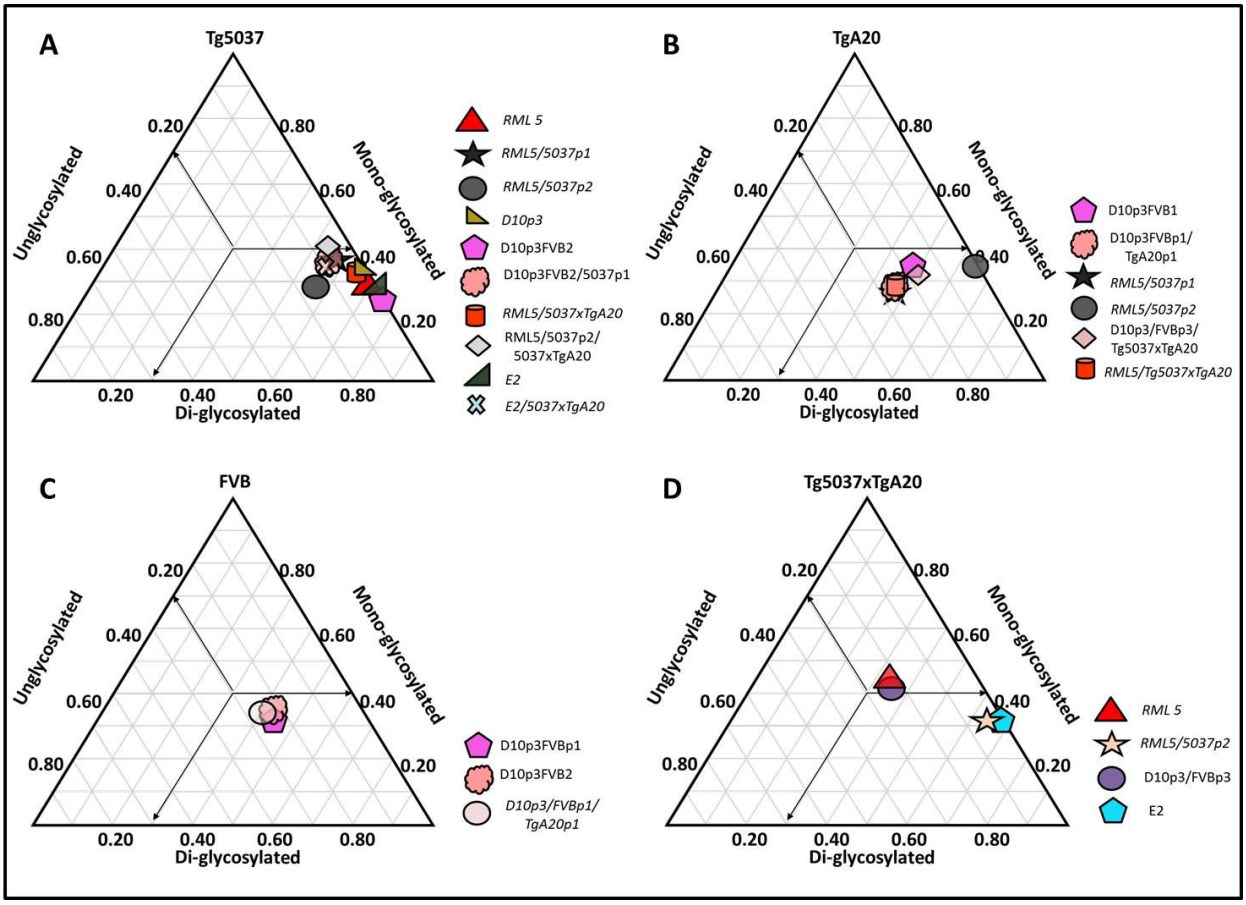

Figure 4.17. Glycoform ratios were analyzed by densiometric analysis by western blot. Ratios are plotted on triplot where arrows indicate the axis that corresponds to the glycoform. The legend to the right of each plot indicates the prion strain while the label at the top of the triplot indicates the mouse strain: **(A)** Tg5037 mice inoculated with mouse and cervid adapted prion strains revealed that primary passage of a strain generated more characteristic CWD glycoform ratios while adaptation of a strain through Tg(5037) mice trended toward an equal di to mono-glycosylated ratio. **(B)** TgA20 and **(C)** FVB mice propagated glycoform ratios characteristic of mouse-adapted prions. **(D)** Primary passage of prion strains in Tg5037xTgA20 mice also generated glycoform ratios characteristic of the donor's PrP^C sequence.

In vitro cervid prion cell culture assay only propagates prion strains from homologous donor

The cervid prion cell assay (CPCA) has been previously reported as an effective *in vitro* assay to investigate species barriers and transmission efficiency between cells expressing heterologous PrP^C and multiple prion strains[39]. Here we used this *in vitro*

system to reproduce our *in vivo* transmission studies with our adapted prion strains and cells expressing mouse or deer PrP^C. This assay revealed more strict species barriers between heterologous PrP^C sequences between donor and host as compared to our bioassay data. Mouse-adapted prion strains were only able to infect cells expressing mouse PrP^C and reciprocally, cervid-adapted prion strains were only able to chronically infect cells expressing cervid PrP^C (Figure 4.18). Our bioassay data contradicts in that RML 5 was able to cause terminal disease in animals that expressed mouse PrP^C, cervid PrP^C, and mice that expressed both.

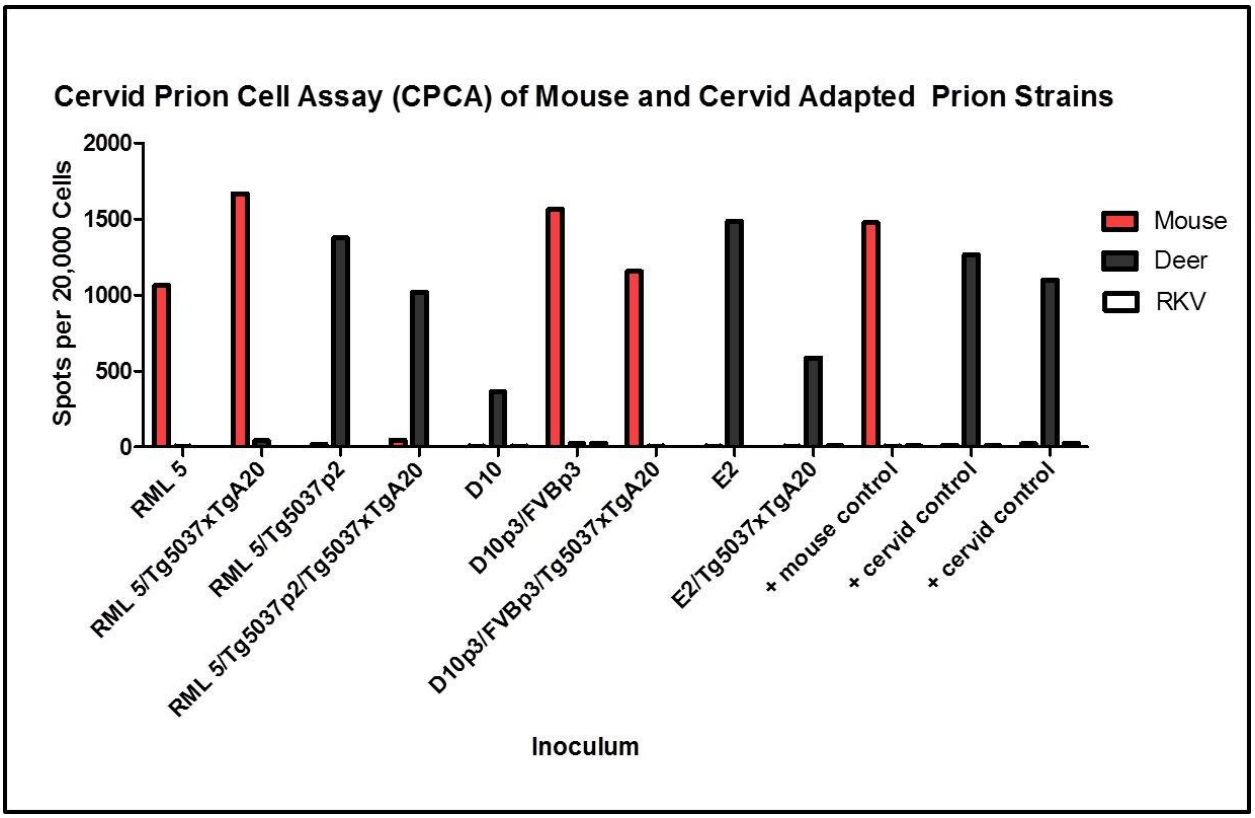

Figure 4.18. Prion strains tested with the Cervid Prion Cell Assay (CPCA) were only able to infect cells expressing homologous PrP^C.

Cervid prion strains propagated through co-overexpressing mice exhibit a decrease in stability while mouse prions become more stable

We conducted a conformational stability assay of our chronically infected cell lines to investigate biochemical differences between strains. 20,000 cells were transferred to elispot plates, incubated with increasing concentrations of guanidine hydrochloride, PK digested, probed with antibody and then analyzed by an elispot plate reader for density. Unexpectedly, we saw differences in conformation stability between mouse and cervid strains that were propagated through Tg5037xTgA20 mice (Figure 4.19). Interestingly, mouse-adapted strains propagated through Tg5037xTgA20 mice had an increase in conformational stability while cervid-adapted strains, notably RML5/5037p2 had a decrease in stability after propagation through co-overexpressing mice (Figure 4.19). Curiously, the RML5/Tg5037xTgA20 strain propagated through a murine cell line had an identical conformational stability as compared to the RML5/5037p2 strain propagated through a cervid cell line (Figure 4.19). Finally, mouse strains that propagated through mouse cell lines had similar conformational stability curves and cervid strains propagated through cervid cell lines grouped together (Figure 4.19).

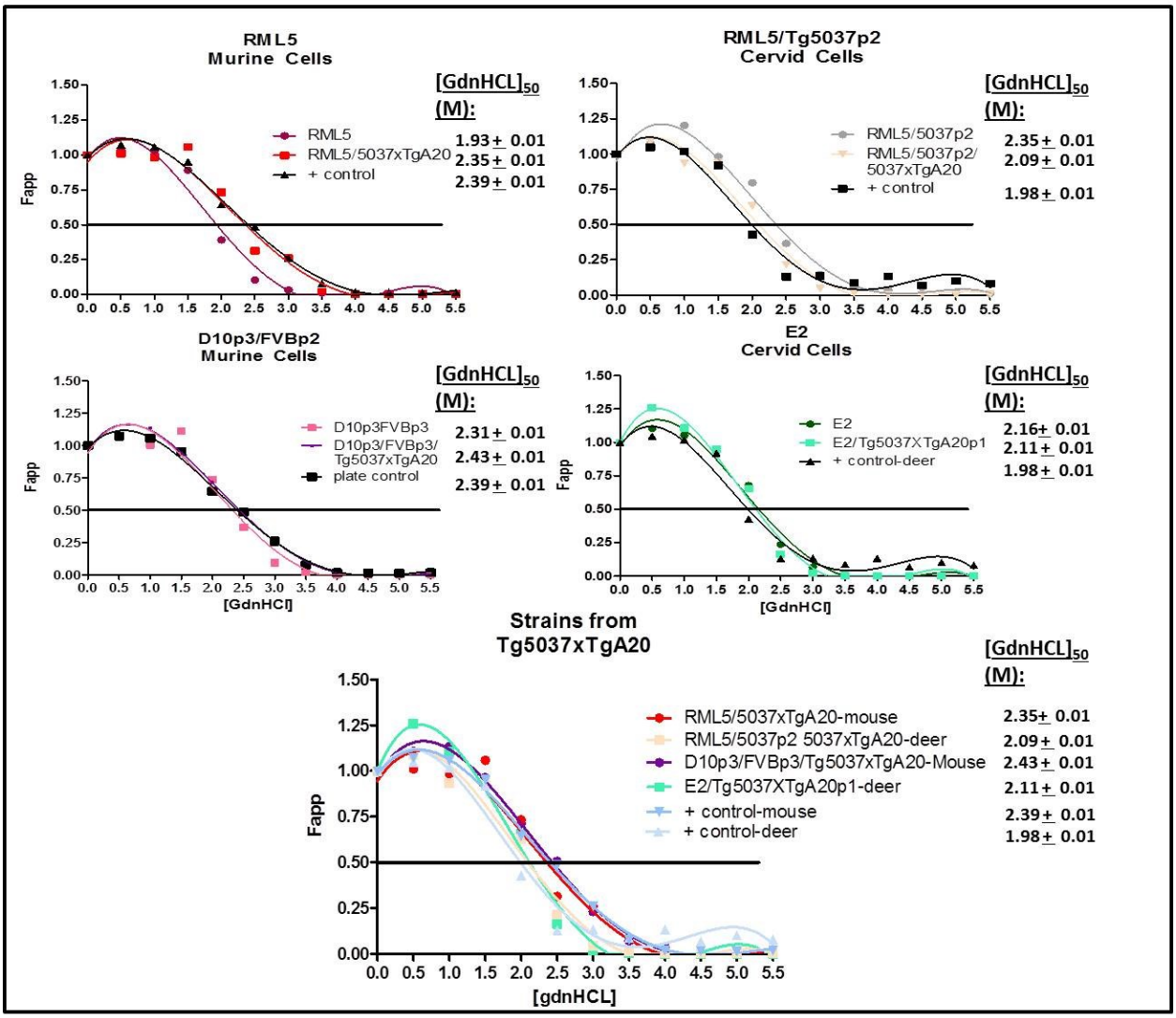

Figure 4.19. Conformation stability curves generated from chronically infected cell lines with mouse and cervid-adapted prion strains. $[GdnHCl]_{50}$ is the concentration required to denature 50% of PrP^{RES}. Mouse-adapted prions adapted through murine cell lines had similar conformational stabilities to each other as did cervid-adapted prions. Interestingly, propagation of mouse-adapted strains through Tg5037xTgA20 mice stabilized strains while the conformational stability of cervid-adapted strains de-stabilized slightly.

DISCUSSION

We have shown for the first time that FVB mice can be susceptible to CWD cervid-adapted prions after subsequent passage of the non-infected brain homogenate

back into FVB mice. We inoculated FVB mice with a CWD strain D10 that was previously propagated through cervidized mice 3 times (D10p3). FVB mice did not show any clinical signs of disease or PK resistant material in brains after 300 dpi. However, subsequent passage of brain homogenate from one of the non-clinical mice led to abrogation of the species barrier and all inoculated mice came down with clinical disease in 153±39 dpi, which is similar to mice inoculated with RML scrapie prions that have been adapted through mice. Other research groups have reported a similar phenomenon in other models. Hill et al., reported subclinical disease in CD-1 wild type mice upon inoculation of 263K hamster scrapie, however, PK resistant material and neuropathology was detected in some of these mice[46]. In contrast, we did not detect PK resistant material in any of the inoculated mice by western blot of IHC. More recently, Castilla et al., demonstrated similar results in a porcine transgenic mouse model inoculated with BSE prions[40]. Brains from inoculated mice without clinical signs of disease and the absence of detectable PK resistant material were able to infect the same mouse model upon subsequent passage. It is possible that the FVB mice we inoculated harbored a very low infectious titer of prion material that was not detectable by conventional methods but was efficiently propagated upon serial passage. However, we accounted for this possibility by inoculating TgA20 mice that overexpress mouse prion protein and have been shown to have a much shorter disease progression compared to wild type mice[34]. Although, we show transmission of the D10p3/FVBp1 strain to TgA20 mice, these mice had a significant delay in disease as compared to TgA20 mice inoculated with RML prions. Additionally, only 3 of the 4 TgA20 mice developed clinical disease. Further passage of the D10p3/FVBp1 strain through TgA20

or FVB mice considerably shortened the incubation period and was stabilized in TgA20 mice after the third passage. The delay in onset to disease of TgA20 mice indicate that prion titers from the primary inoculum were not the reason we did not see clinical signs in FVB mice. It does suggest, however, that a new strain emerged as TgA20 mice with the same PrP^{C} primary sequence as FVB mice, albeit in higher amounts, did not succumb to prion disease as rapidly as other reported mouse-adapted strains. These differences could be attributed to the difference in mouse backgrounds and if this is true, this point validates our hypothesis that other host factors, in addition to PrP^{C} sequence, play a role in strain adaptation. Curiously, FVB mice inoculated with D10 (natural isolate of deer CWD before adaptation through cervidized mice) also did not show clinical signs of disease or detectable PrP^{RES} and subsequent passage through FVB mice, mimicking the previous experimental design, also did not succumb to disease. It is plausible that abrogation of the murine species barrier with CWD prions requires additional host factors present in the mice.

To further assess characteristics of the newly generated mouse-adapted CWD strain we passaged the brain homogenate from terminally sick FVB mice and brain homogenate from the primary passage of D10p3/FVBp1 from TgA20 mice back into the original host, Tg(5037) mice expressing cervid prion protein. Surprisingly, Tg(5037) were 75% susceptible to the mouse-adapted strain with 30 day increase in incubation as compared to the FVB mice inoculated with the same strain. An additional passage into Tg(5037) mice shortened the incubation period and had a complete attack rate. In contrast, Tg(5037) mice inoculated with the CWD adapted-TgA20 strain were completely resistant to infection. It is possible that prion titers were lower in the TgA20

brain homogenate because these mice succumb to disease in a shorter time period giving less time for replication of infectious prions. We tested this theory by inoculating the TgA20-adapted CWD strain back into FVB mice. Consequently, all FVB mice were clinical at 155 dpi. This suggests that the overexpression of mouse prion protein in TgA20 mice had a role in generating a new species barrier. Perhaps, as Kimberlin has reported, multiple conformers are present in brain homogenate of infected animals and the overexpression of mouse PrP^C in TgA20 mice allows for a more efficient selection and propagation of a mouse prion conformer and conformers that would transmit readily to cervidized mice are diluted out[15]. Interestingly, passage of the D10p3/FVBp2/Tg5037p1 strain into TgA20 mice generated another species barrier as TgA20 mice were completely resistant to infection. Perhaps providing additional evidence that the FVB brain homogenate contained more than one strain and those strains were preferentially replicated by different host PrP^C sequences, diluting out the other infectious strains so that transmission is now barred in heterologous species.

Remarkably, Tg(5037) mice were 75% susceptible to mouse-adapted RML 5 strain upon primary passage and they exhibited an incubation period identical to FVB mice inoculated with the same strain. Previous reports have demonstrated a strict species barrier between mouse-adapted prions and cervidized mice[24,25, 2627, 28]. However, most of these studies report on a cervid transgenic line that expresses deer prion protein. Here we report efficient transmission in elk cervid transgenic mice. It is possible that transmission barrier differences can be attributed to differences at amino acid 226 in deer and elk PrP^C primary sequence, although the mechanism is unclear[17]. Further adaptation of RML through Tg(5037) shortened the stabilized the incubation period.

Curiously, tertiary passage through Tg(5037) mice drastically increased the incubation period but this result from not using a pooled inoculum from all mice and using a mouse that developed disease in between other mice. Passage of the newly generated cervid-adapted RML prions (RML5/5037p1) into TgA20 mice caused disease in all of the mice within 80 dpi. This was a much shorter incubation compared to TgA20 mice inoculated with the mouse-adapted CWD strain from FVB mice. However, RML 5 adapted through Tg(5037) mice twice (RML5/5037p2) generated a species barrier between TgA20 mice. It is important to note that one of the TgA20 mice did show clinical signs and PK resistant material in the brain after inoculation of RML5/5037p2. Even more importantly is that the PK resistant material from this mouse was only detected with a cervid specific antibody 9E9 and glycoform ratios revealed predominately di-glycosylated banding pattern, a characteristic of CWD prions (Figure 4.17). Unfortunately, based on other reports of spontaneous generation of prions in overexpressing transgenic mouse models[40,47], we cannot rule out this possibility, however, you would not expect to see spontaneous mouse prions detectable with a cervid specific antibody. Further analysis and experimentation would be necessary to confirm this result.

We further investigated strain properties of our mouse and cervid-adapted prions by bioassay into Tg5037xTgA20 mice that co-overexpress mouse and cervid prion protein. We expected inhibition of transmission in these mice because of the heterologous expression of PrP^{C}. However, Tg5037xTgA20 mice were susceptible, with varying degrees, to all 4 strains we challenged them with. Primary passage of mouse-adapted prion strains RML 5 and D10p3/FVBp3 preferentially propagated mouse prions and the inverse was demonstrated by cervid-adapted RML 5 and E2 (CWD natural

isolate) as they preferentially propagated cervid prion strains. Passage of RML 5 through co-overexpressing mice developed disease in 93 dpi, which is an intermediate time between TgA20 mice inoculated with RML 5 (60dpi) and Tg(5037) mice (158dpi). The D10p3/FVBp3 strain produced disease in slightly shorter time frame of 83 dpi but also had a complete attack rate. In contrast, cervid-adapted RML and E2 inoculated into Tg5037xTgA20 showed a delay in disease at 216 and 265 dpi respectively and E2 only infected 4/5 mice. Differences in incubation times and attack rates could be attributed to differences in cervid to mouse PrP^C expression levels in these mice but western blot analysis using discriminatory antibodies suggest that expression is similar in most cases (Figure 4.9B). When mice were screened for co-expression of cervid and mouse PrP^C by PCR, we did detect some littermates that only expressed mouse or cervid prion protein. We utilized these mice as mouse only or cervid only controls and we found that the controls were consistent with bioassay results attained from TgA20 and Tg(5037) mouse strains. Our mouse only controls were only susceptible to mouse-adapted strains and were infected with similar incubation periods to TgA20 mice inoculated with RML 5 and the same was demonstrated with our cervid only controls as they were susceptible to all the strains as previously described. Finally, we inoculated strains propagated through Tg5037xTgA20 mice back into the original host to investigate the generation of new species barriers and new strains. Interestingly, mouse-adapted prions, including mouse-adapted CWD did transmit to TgA20 mice with great efficiency in 67 and 69 dpi respectively. These strains also transmitted to Tg(5037) mice but with varying efficiency. RML 5/Tg5037xTgA20 propagated in Tg(5037) mice with an increased incubation time similar to that observed with RML5/5037p2 passaged through

Tg5037xTgA20 mice of 213 dpi. The D10p3/FVBp3/Tg5037xTgA20 strain infected 3 of 5 Tg(5037) mice with an identical incubation period as seen with RML5/Tg5037xTgA20. In contrast, neither of the cervid-adapted strains propagated through Tg5037xTgA20 mice transmitted to TgA20 mice. This suggests that cervid prion protein is more promiscuous and more easily converted to a disease causing isoform. Several groups have now show that the rigid loop conformation of cervid prion protein increases transmission efficiency and has a higher propensity to misfold[45,48-50]. We feel our bioassay data strongly correlates with other reports. It is plausible that the rigid loop structure actually allows for more flexibility of misfolding, almost by creating a structural platform that allows heterologous PrP^{RES} to interact and dictate conformational changes more easily.

Biochemical and histological analysis provide further evidence that cervid prion protein is promiscuous. Tg(5037) mice and Tg5037xTgA20 mice exhibited a great degree of neuropathology as compared to TgA20 and FVB mice. Interestingly, IHC of Tg5037xTgA20 mice inoculated with cervid-adapted strains exhibited PrP^{RES} deposition with both mouse and cervid prion characteristics. It is important to note that is was difficult to detect PrP^{RES} by IHC in FVB, TgA20, and Tg5037xTgA20 mice that were inoculated with the mouse-adapted CWD strain, D10p3/FVBp3 despite detectable amounts by western blot. We feel that this strain may have become acid sensitive, a harsh step in our IHC protocol. Further analysis should be done to interpret these results adequately.

Glycoform ratio analysis demonstrated that the host PrP^{C} of inoculated animals corresponded with strain characteristics for mice. TgA20 and FVB mice propagated

strains that had higher mono-glycosylated to di-glycosylated ratios and increased un-glycosylated material. In contrast, Tg(5037) mice did not produce consistent glycoform ratios. Primary passage of a heterologous strain usually produced more di-glycosylated prions but serial passage lowered the di to mono-glycosylated ratio to more equal values. Perhaps this is further evidence that cervid PrP^C is more impressionable.

Finally, *in vitro* experimentation utilizing CPCA to propagate adapted strains in cervid or murine PrP^C expressing lines demonstrated that transmission was only efficient between homologous sequences, i.e., cervid-adapted prions could only infect cervid cells and vice- versa. This was in contrast to our bioassay data where Tg(5037) mice could propagate mouse or cervid-adapted prions (with varying efficiency). This also provides evidence that certain host factors are required for strain adaptation. Conformational stability assays of infected cell lines revealed that mouse-adapted prions similarly increased in stability after passage through Tg5037xTgA20 mice whereas cervid-adapted prions slightly decreased in stability. Interestingly, $GdnHCl_{50}$ curves for mouse-adapted prions were similar demonstrating that CWD was efficiently adapted through a murine host. Intriguingly, the RML5 strain adapted through Tg5037 mice twice had the same conformational stability as RML5 propagated through Tg5037xTgA20 mice. However, the RML5/Tg5037p2 strain dramatically reduced in stability once propagated through Tg5037xTgA20 mice. A possible explanation is that even though cervid prions are not propagated in Tg5037xTgA20 mice inoculated with RML5 (at least not at detectable levels), cervid PrP^C may interact and play a role in mouse PrP^C conversion.

We have demonstrated through extensive bioassay experiments that we were able to adapt CWD through FVB mice after adaptation of CWD through cervidized mice. Additionally, we have collectively shown that cervid prion protein is more promiscuous and more readily converted to an infectious prion no matter the host's primary PrP^C sequence. We have also demonstrated that cervid prion protein promiscuity may rely on additional host factors for efficient transmission and adaptation. Further investigations on the mechanisms involved in converting cervid prion protein could help lead to therapeutic strategies that could aid in generating transmission and species barriers so prion replication would be terminated.

REFERENCES

1. Williams, E. S., McCarty, C. & Spraker, T. Epizootiology of chronic wasting disease in free-ranging cervids in Colorado and Wyoming. *of Wildlife Diseases* (2000).

2. Williams, E. S. & Young, S. SPONGIFORM ENCEPHALOPATHY OF ROCKY MOUNTAIN ELK 1. *Journal of wildlife diseases* (1982).

3. Prusiner, S. B. Novel proteinaceous infectious particles cause scrapie. *Science (New York, N.Y.)* **216,** 136-44 (1982).

4. Cohen, F. E. *et al.* Structural clues to prion replication. *Science (New York, N.Y.)* **264,** 530-1 (1994).

5. Pan, K., Baldwin, M. & Nguyen, J. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. in *Proceedings of the* (1993).

6. Pattison, I. & Millson, G. Scrapie produced experimentally in goats with special reference to the clinical syndrome. *Journal of Comparative Pathology and* (1961).

7. Pattison, I. H. Scrapie in the welsh mountain breed of sheep and its experimental transmission to goats. *The Veterinary record* **77,** 1388-90 (1965).

8. Telling, G. C. Transgenic mouse models and prion strains. *Prion Proteins* (2011).

9. Raymond, G., Bossers, A. & Raymond, L. Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. *The EMBO* (2000).

10. Hill, A. & Collinge, J. Prion strains and species barriers. *2004* (2004).

11. Horiuchi, M., Priola, S. & Chabry, J. Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers. in *Proceedings of the* (2000).

12. Scott, M., Groth, D., Tatzelt, J. & Torchia, M. Propagation of prion strains through specific conformers of the prion protein. *Journal of* (1997).

13. Safar, J. *et al.* Eight prion strains have PrPSc molecules with different conformations. *Nature medicine* (1998).

14. Peretz, D., Scott, M., Groth, D. & Williamson, R. A. Strain-specified relative conformational stability of the scrapie prion protein. *Protein* (2001).

15. Kimberlin, R. H. & Walker, C. A. Evidence that the Transmission of One Source of Scrapie Agent to Hamsters Involves Separation of Agent Strains from a Mixture. *Journal of General Virology* **39,** 487-496 (1978).

16. Kimberlin, R. H. *et al.* Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. *The Journal of general virology* **68 (Pt 7),** 1875-1881 (1987).

17. Angers, R. C. *et al.* Prion Strain Mutation Determined by Prion Protein Conformational Compatibility and Primary Structure. *Science* **328,** 1154-1158 (2010).

18. Prusiner, S. B. *et al.* Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63,** 673-86 (1990).

19. Kimberlin, R. H. & Walker, C. A. Pathogenesis of scrapie: agent multiplication in brain at the first and second passage of hamster scrapie in mice. *Journal of General Virology* (1979).

20. Scott, M. *et al.* Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell* **59,** 847-857 (1989).

21. Torchia, M., Cohen, F. E., DeArmond, S. & Prusiner, S. B. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* (1995).

22. Prusiner, S. B. *et al.* Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63,** 673-686 (1990).

23. Telling, G. C., Haga, T., Torchia, M. & Tremblay, P. Interactions between wild-type and mutant prion proteins modulate neurodegeneration in transgenic mice. *Genes &* (1996).

24. Browning, S. R. *et al.* Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *Journal of* 13345-13350 (2004).

25. Meyerett, C. *et al.* In vitro strain adaptation of CWD prions by serial protein misfolding cyclic amplification. *Virology* **382,** 267-276 (2008).

26. Favara, C., Gardner, D. & Williams, E. S. Transmission and adaptation of chronic wasting disease to hamsters and transgenic mice: evidence for strains. *Journal of* (2007).

27. Williams, E. S. & Young, S. Spongiform encephalopathies in Cervidae. *Revue scientifique et technique (* (1992).

28. Kurt, T., Telling, G. C., Zabel, M. & Hoover, E. Trans-species amplification of PrP CWD and correlation with rigid loop 170N. *Virology* 1-9 (2009).

29. Mathiason, C., Carver, S., Zabel, M. & Telling, G. C. Detection of chronic wasting disease prions in salivary, urinary, and intestinal tissues of deer: potential mechanisms of prion shedding and transmission. *Journal of* (2011).

30. Mathiason, C., Hays, S., Powers, J. & Hayes-Klug, J. Infectious prions in pre-clinical deer and transmission of chronic wasting disease solely by environmental exposure. *PLoS One* (2009).

31. Williams, E. S. & Miller, M. W. Chronic wasting disease in deer and elk in North America. *Revue scientifique et technique (* (2002).

32. Tamgüney, G., Miller, M. W., Wolfe, L. & Sirochman, T. Asymptomatic deer excrete infectious prions in faeces. *Nature* (2009).

33. Spraker, T., VerCauteren, K. & Gidlewski, T. Antemortem Detection of PrPCWD in Preclinical, Ranch-Raised Rocky Mountain Elk (Cevvus Elaphus Nelsoni) by Biopsy of the Rectal Mucosa. *Journal of Veterinary* (2009).

34. Fischer, M., Rülicke, T., Raeber, A. & Sailer, A. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *The EMBO* (1996).

35. Angers, R. C. *et al.* Chronic Wasting Disease Prions in Elk Antler Velvet. *Emerging Infectious Diseases* **15,** 696-703 (2009).

36. Calvi, C. *et al.* Characterization of Conformation-dependent Prion Protein Epitopes. *Journal of Biological Chemistry* **287,** 37219-37232 (2012).

37. Williamson, R. A. *et al.* Mapping the Prion Protein Using Recombinant Antibodies. *Journal of Virology* **72,** 9413-9418 (1998).

38. Prusiner, S. B., Hadlow, W. J., Eklund, C. M., Race, R. E. & Cochran, S. P. Sedimentation characteristics of the scrapie agent from murine spleen and brain. *Biochemistry* **17,** 4987-92 (1978).

39. Bian, J., Khaychuck, V., Angers, R., Graham, C. & Telling, G. Cell-Based Quantification of Chronic Wasting Disease Prions. *Journal of Virology* **84,** 8322-8326 (2010).

40. Castilla, J. Subclinical Bovine Spongiform Encephalopathy Infection in Transgenic Mice Expressing Porcine Prion Protein. *Journal of neuroscience* **24,** 5063-5069 (2004).

41. Bartz, J., Kramer, M., Sheehan, M. & Hutter, J. Prion interference is due to a reduction in strain-specific PrPSc levels. *Journal of* (2007).

42. Bartz, J., Aiken, J. & Bessen, R. Delay in onset of prion disease for the HY strain of transmissible mink encephalopathy as a result of prior peripheral inoculation with the replication-deficient DY strain. *Journal of General Virology* (2004).

43. Dickinson, A. G., Fraser, H. & Outram, G. Scrapie incubation time can exceed natural lifespan. *Nature* (1975).

44. Kimberlin, R. & Walker, C. Competition between strains of scrapie depends on the blocking agent being infectious. *Intervirology* (1985).

45. Tamgüney, G. *et al.* Chimeric elk/mouse prion proteins in transgenic mice. *Journal of General Virology* **94,** 443-452 (2013).

46. Hill, A. F., Joiner, S. & Linehan, J. Species-barrier-independent prion replication in apparently resistant species. in *Proceedings of the* (2000).

47. Westaway, D., DeArmond, S. & Cayetano-Canlas, J. Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild-type prion proteins. *Cell* (1994).

48. Sigurdson, C. J., Nilsson, K. P. R. & Hornemann, S. A molecular switch controls interspecies prion disease transmission in mice. *The Journal of clinical* (2010).

49. Kyle, L. M., John, T. R., Schätzl, H. M. & Lewis, R. V. Introducing a Rigid Loop Structure from Deer into Mouse Prion Protein Increases Its Propensity for Misfolding In Vitro. *PLoS One* **8,** e66715 (2013).

50. Sigurdson, C. J. *et al.* Spongiform Encephalopathy in Transgenic Mice Expressing a Point Mutation in the 2- 2 Loop of the Prion Protein. *Journal of neuroscience* **31,** 13840-13847 (2011).

OVERALL CONCLUSION AND FUTURE DIRECTIONS

The ability of prions to encode strain properties in the absence of nucleic acid has confounded fundamental biological principles by demonstrating the significance of protein folding and conformation to epigenetic information transfer and infection. Discoveries elucidating mechanisms of strain adaptation and species barriers are important for developing therapeutic strategies for diseased patients and for abrogating efficient inter and intra species transmission. However, lengthy incubation periods and enigmatic strain properties have proved challenging to study prion strain characteristics. The use of *in vitro* methods, like cell culture, to study prion disease have led to expedited models of prion disease but often lack biological relevance. Although transgenic rodent models mimic natural prion infection and have allowed researchers to further exploit prion disease pathology, disease incubation periods can be extraordinarily long. Combining *in vivo* and *in vitro* assays can provide insight into prion replication, pathology, and strain mechanisms. The invention of PMCA has provided a biologically relevant tool for elucidating prion transmission and replication mechanisms within days to weeks rather than months and years.

The first part of this dissertation we assessed the ability of PMCA to adapt CWD prions *in vitro* as compared to *in vivo* mouse bioassay. Here we report efficient linear amplification of PrP^{CWD} by sPMCA resulting in *in vitro* generation of infectious CWD prions. Remarkably, we observed a drastic, nearly identical reduction in incubation time to terminal disease of CWD-susceptible Tg(cerPrP)1536 mice inoculated with *in vitro*-amplified or mouse-passaged prions from the D10 isolate of CWD prions when

compared to the original D10 inoculum. *In vitro*-amplified and *in vivo*-passaged D10 also shared similar neuropathological and biochemical characteristics that were significantly different than the original D10 strain, more closely resembling the RML strain of mouse-adapted scrapie prions. By all accepted parameters used to characterize prion strains that we examined, sPMCA adapted the D10 CWD strain as efficiently as passage in Tg(cerPrP)1536 mice, and represents a powerful, efficient tool for assessing strain adaptation and species barriers *in vitro*.

We and others observed that PMCA could generate *de novo* prions. We subjected uninfected brain homogenate derived from cervidized transgenic mice to seven rounds (48 cycles each) of PMCA under normal conditions in a prion-free laboratory using all new reagents and equipment. Bioassay determined that the spontaneously generated cervid prions were infectious to cervidized transgenic but not wild type mice and biochemical analysis resulted in a unique profile differing from prion strains used within our laboratory. These data strongly suggest that these prions were generated spontaneously and not from contamination. We next examined the rate of spontaneous conversion with our PMCA protocol. We generated PK-resistant *de novo* prions that were detectable by western blot at round 4 of sPMCA at a rate of 1.6%. Further generation of spontaneous prions were observed after round 5 and round 7 with rates of 5.0% and 6.74% respectively. These low rates of spontaneous conversion support PMCA as an efficient and sensitive tool to investigate strain adaptation and species barriers if limited rounds and proper controls are used.

Many research groups have identified a species barrier in wild type mice. In particular, wild type mice appear resistant to CWD prions. Curiously, the data reported in the

second chapter of this dissertation suggested that adaptation of cervid prions through cervidized mice resulted in strain properties resembling mouse-adapted prion strains. Therefore, we hypothesized that mouse host factors may play a role in adaption in conjunction with host encoded PrP^C and this adaptation could lead to abrogation of species barriers. We challenged this hypothesis by mouse bioassay and reported for the first time adaption of cervid prions through wild type mice after secondary passage. Because primary passage of cervid adapted prions did not cause clinical disease, we further investigated the propensity of cervid PrP^C misfolding and the role host co-factors may play in strain adaptation and transmission efficiency. We challenged mice overexpressing murine PrP^C, wild type mice, and Tg5037xTgA20 mice that co-overexpress cervid and murine PrP^C with mouse and cervid-adapted prion strains. Biological and biochemical characterization revealed successful propagation of mouse-adapted and cervid-adapted strains through cervidized mice. We observed limited propagation of cervid-adapted prions through hosts expressing only murine PrP^C. Passage of mouse and cervid adapted strains through mice co-overexpressing mouse and cervid prion protein revealed that homologous strains were preferentially generated. Re-passage of the co-adapted strains through mice expressing only cervid or mouse PrP^C divulged species barriers between cervid prions and mice expressing murine PrP^C while mice expressing cervid PrP^C converted both mouse and cervid adapted strains with varying degrees of efficiency. We conclude that cervid prion protein can interact more promiscuously with heterologous prion strains and, therefore, abrogates the species barrier between cervid and mouse prion protein.

We are very interested in continuing this work *in vitro* using PMCA to further elucidate the mechanisms of prion strain adaptation and abrogation of species barriers. We propose to further test the promiscuity of cervid PrP^C to convert various prion strains by utilizing mice that transgenically express the rigid loop region of cervid PrP^C as a substrate for PMCA. This experiment would also allow us to test the effect of murine host factors in the presence of a rigid loop. It would be interesting to see if species barriers are abrogated solely by the replacement of the murine loop with a cervid loop or if other factors play a role in adaptation and promiscuity. Similarly, it would be intriguing to further test strain adaptation of our newly generated mouse and cervid-adapted prions in these same transgenic mice through mouse bioassay.

Additionally, we would like to investigate the phenomenon of propagating heterologous prion strains. As seen in figure 4.6, we rarely identified a heterologous prion strain detected using mouse and cervid PrP^C discriminatory antibodies. Further analysis of these samples by genotyping, bioassay, PMCA, and western blot would confirm that the prion strain was truly heterologous to host PrP^C.

Investigation of strain adaptation and species barrier through cell culture models such as CPCA could help determine host factor requirements for conversion. The differing results of infection exhibited between bioassay and CPCA suggest that host factors that are absent in RK13 cells are needed to abrogate species barriers. We hope to further test this hypothesis by using cell from different origins in addition to using less adapted cervid and mouse prion strains (passaged through the host once rather than two or three times).

Finally, we think it would be important to conduct competitive bioassay experiments using a mixture of cervid and mouse adapted strains and propagating these through mouse only, cervid only, or mouse and cervid expressing mice. Probing the resultant strains propagated with cervid and mouse discriminatory antibodies would identify if cervid, mouse or both prion strains would be preferentially propagated. We also plan to use these discriminatory antibodies with IHC to determine if mouse and cervid prions co-localize in brain areas of co-expressing mice.

In conclusion, our research has showed the importance of host factors, in addition to host encoded PrP^C, for strain adaptation. We have also provided further evidence that cervid PrP^C promiscuity can abrogate species barriers and lead to efficient inter and intra species transmission. As we gain more knowledge about the requirements for host adaptation and transmission the prion field will have more insight into generating therapeutic strategies for infected individuals and ultimately, investigators can determine the tools necessary for eliminating transmission of prion disease.

APPENDIX

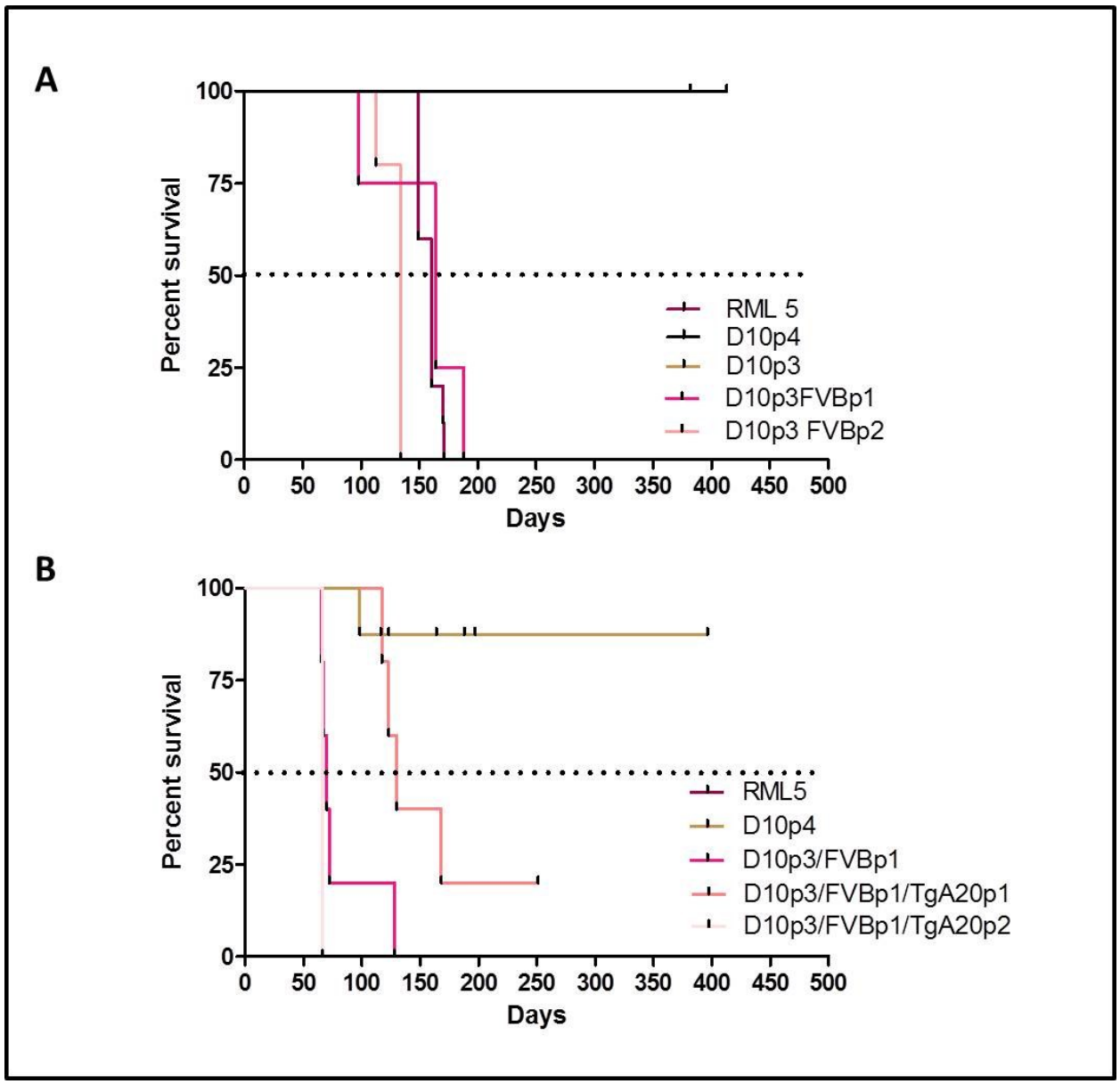

Figure A1.1. Survival curves of **(A)** FVB wild type mice and **(B)** TgA20 mice overexpressing murine prion protein inoculated with cervid-adapted CWD prions as compared to mouse-adapted RML 5. FVB and TgA20 are both resistant to primary passage of CWD prions adapted through cervidized mice but are susceptible upon secondary passage through FVB mice. The legend indicates the inoculum used to produce the survival curves.

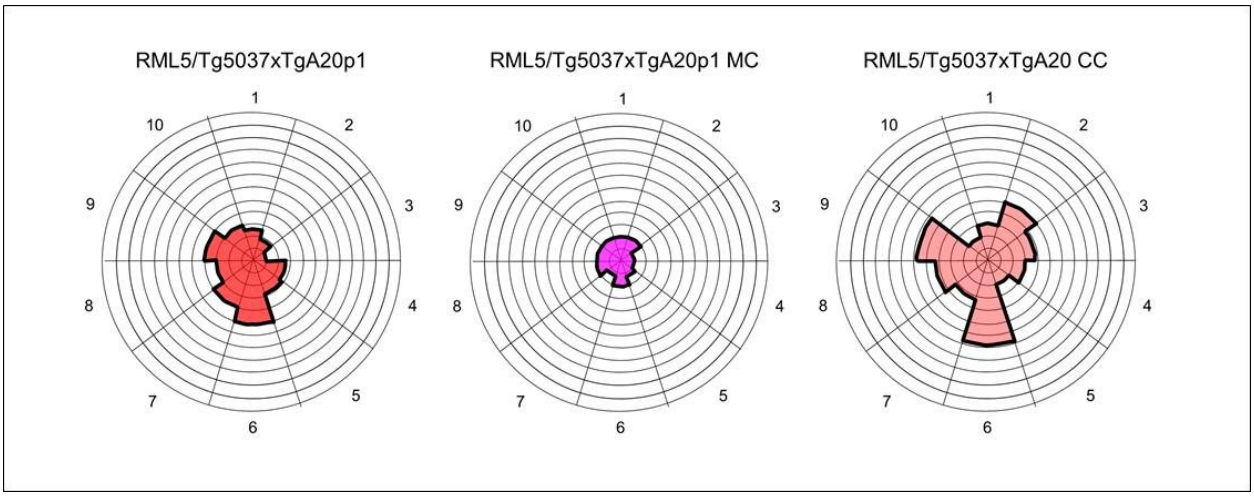

Figure A1.2. Radar plots plotting neuropathology of Tg5037xTgA20 mice co-overexpressing mouse and cervid prion protein and controls. MC= mouse control; this mouse only expresses mouse prion protein and CC= cervid control; this mouse only expresses cervid prion protein. Neuropathology is less severe in mouse models expressing mouse PrP^{C} but is moderate in mice expressing only cervid prion protein. Some of this difference could be attributed to the absence of detectable PrP^{RES} by IHC. We presume that the mouse-adapted prion strains have become sensitive to the acid treatment step in our IHC protocol.

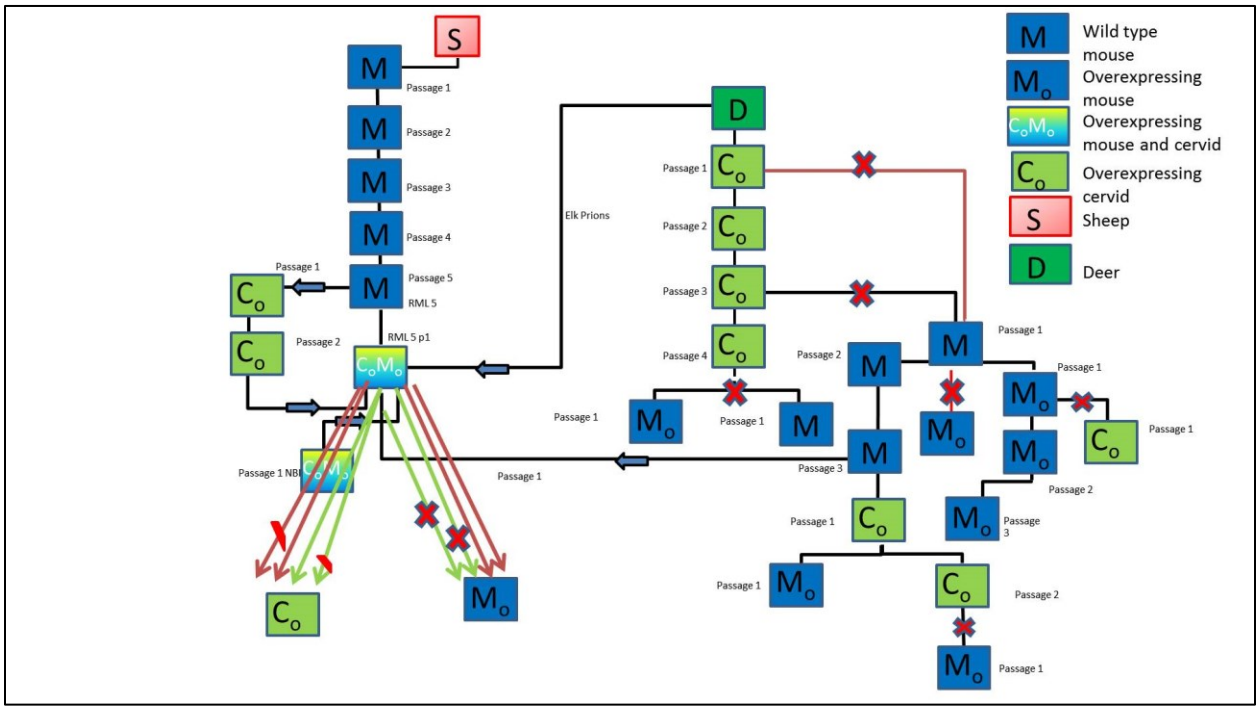

Figure A1.3. Bioassay flowchart showing the generation steps of each strain. Passage indicates how many times the inoculum has been propagated through that mouse strain and X indicate that there was no clinical disease in those animals and a \ indicates

incomplete attack rates. Notice that non-transmission events usually are associated with mouse prion protein expressers and not cervid.

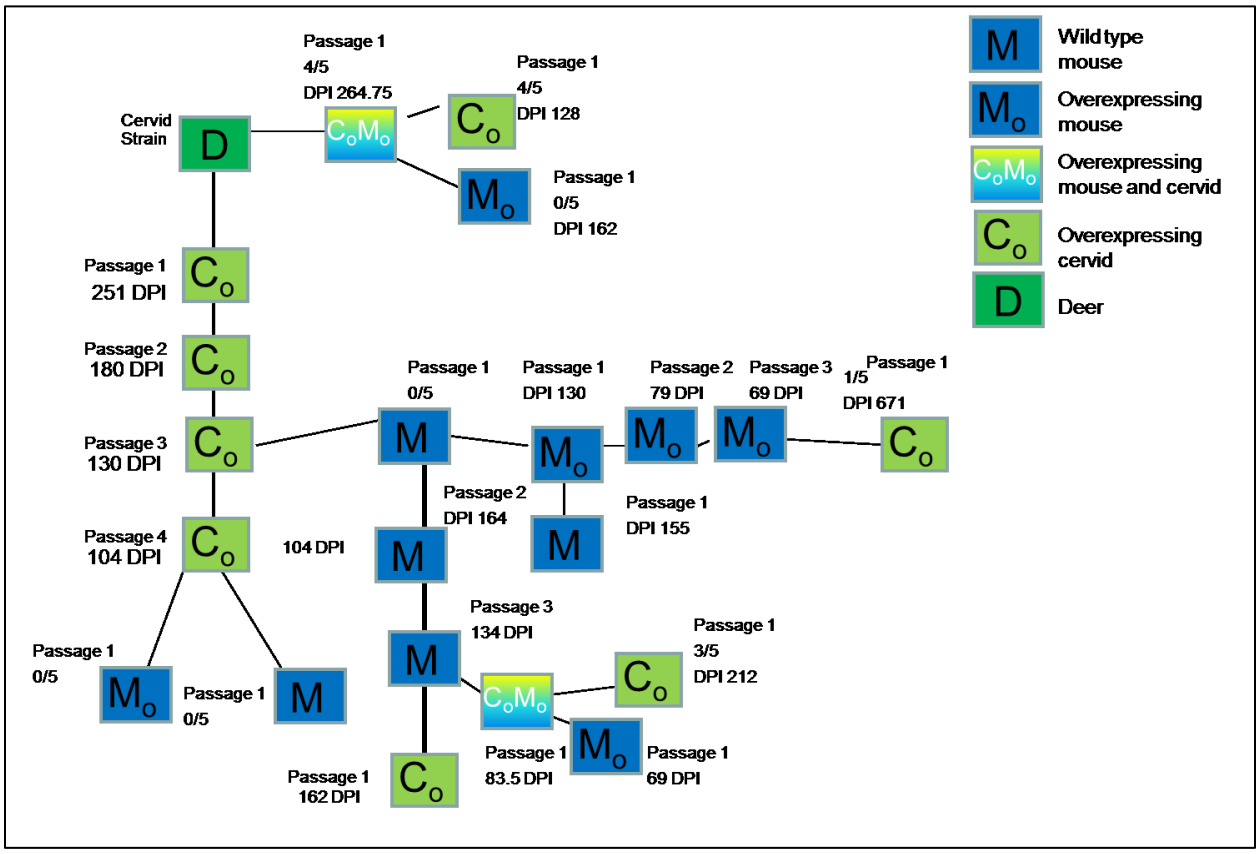

Figure A1.4. Prionogenic tree off mouse-adapted CWD prions. Branch lengths indicate relatedness of strain to host as determined by incubation periods.

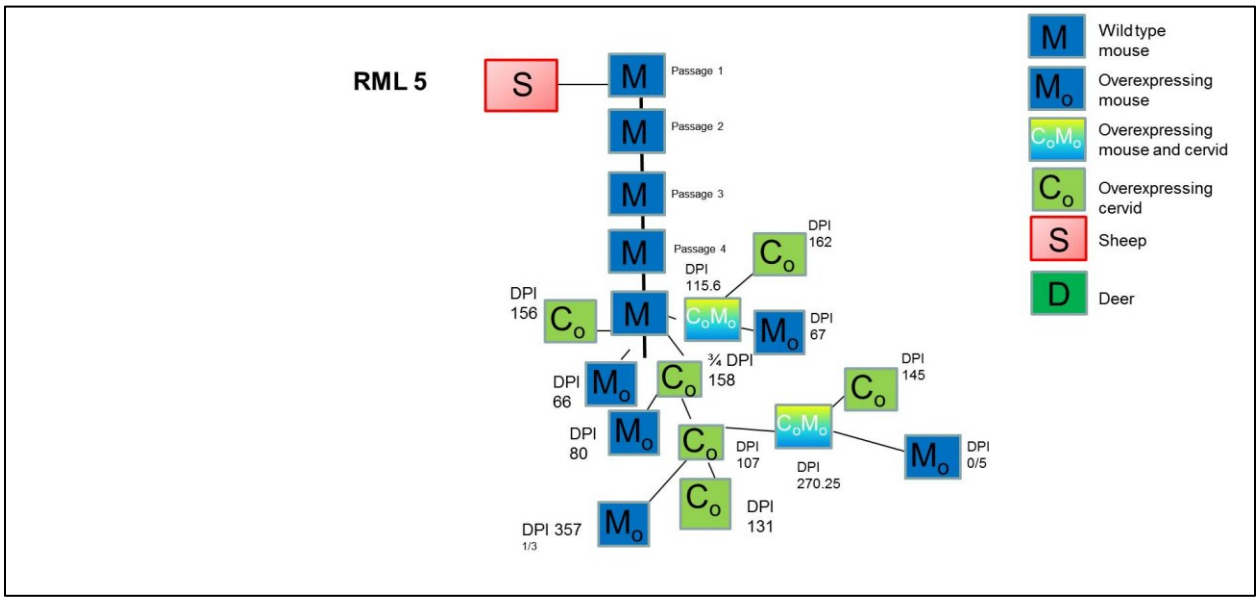

Figure A1.5. Prionogenic tree of cervid-adapted RML 5 prions. Branch lengths indicate relatedness of strain to host based upon incubation periods.

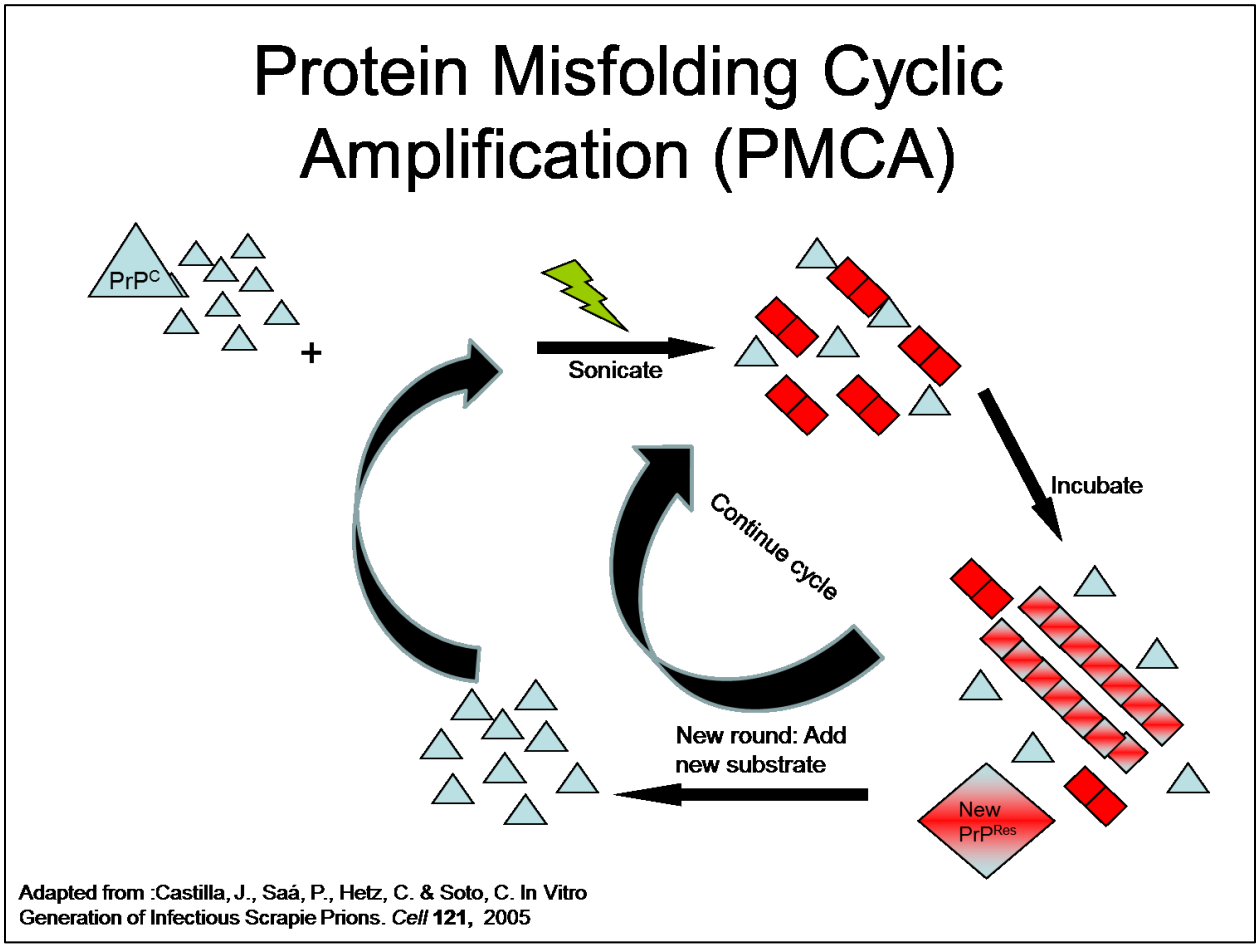

Figure A1.6. Illustration of PMCA protocol used for Chapter 3. Notice there is no addition of a prion seed, just normal brain homogenate (NBH) used as a PrP^C substrate.