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# Cell biology of prion strains in vivo and in vitro

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## Abstract

The properties of infectious prions and the pathology of the diseases they cause are dependent upon the unique conformation of each prion strain. How the pathology of prion disease correlates with different strains and genetic backgrounds has been investigated via *in vivo* assays, but how interactions between specific prion strains and cell types contribute to the pathology of prion disease has been dissected more effectively using *in vitro* cell lines. Observations made through *in vivo* and *in vitro* assays have informed each other with regards to not only how genetic variation influences prion properties, but also to how infectious prions are taken up by cells, modified by cellular processes and propagated, and the cellular components they rely on for persistent infection. These studies suggest that persistent cellular infection results from a balance between prion propagation and degradation. This balance may be shifted depending upon how different cell lines process infectious prions, potentially altering prion stability, and how fast they can be transported to the lysosome. Thus, *in vitro* studies have given us a deeper understanding of the interactions between different prions and cell types and how they may influence prion disease phenotypes *in vivo*.

## Introduction

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a family of diseases with wide ranging strain dependent pathologies (Aguzzi et al., 2007) caused by the deposition of infectious prions in tissue (Beekes et al., 2007) and subsequent interference with vital cellular processes, primarily in the brain (Harris et al., 2006). Infectious prions, known as $PrP^{Sc}$, exist almost exclusively as insoluble and partially protease resistant aggregates of non-native prion protein that propagate via a process of seeded polymerization whereby the native, soluble, and protease-sensitive conformation of cellular prion protein, known as $PrP^{C}$, interacts with $PrP^{Sc}$ and is converted into $PrP^{Sc}$ with the same non-native conformation (Collinge et al., 1996). $PrP^{Sc}$ is found in several cellular compartments along the endocytic pathway where conversion occurs, including early endosomes and endolysosomes, and accumulates in lysosomes (Priola, 2018). In prion disease, $PrP^{Sc}$ is the main, and possibly sole, component of the infectious prion (Collinge et al., 1996). While this conversion happens without guidance from coding nucleic acid (Prusiner, 1982), genetic polymorphism in the prion protein gene PRNP can still play a critical role in prion disease as

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point mutations in $PrP^C$ can alter prion infectivity and prion disease pathogenesis (Parchi et al., 1996).

A wide range of non-native conformations in the prion protein are infectious and able to promote the conversion of $PrP^C$ into their own distinctive $PrP^{Sc}$ conformation. This variation in $PrP^{Sc}$ conformation leads to infectious prions with different properties that may explain the existence of prion strains, which are defined *in vivo* by variations in prion disease presentation and pathology. The unique conformational structure adopted by $PrP^{Sc}$ leads to its ordered assembly into large aggregates, the properties of which likely contribute to the prion disease phenotypes (Morales et al., 2007). For example, the structure of $PrP^{Sc}$ aggregates (Kraus et al., 2021) are believed to contribute to the specific and reproducible pathologies associated with different prion strains (Bessen et al., 1992; Caughey et al., 1998; Parchi et al., 1996; Spassov et al., 2006), the cellular uptake routes that best facilitate prion propagation (Fehlinger et al., 2017), the rates of prion degradation by cells (Choi et al., 2013; Shoup et al., 2021), and the susceptibility of different cells to prion infection (Nishida et al., 2000). While the phenotypic differences between strains of prions is thought to primarily be the result of conformational differences in $PrP^{Sc}$, post-translational modifications to $PrP^C$ may alter the phenotypic properties of prion strains, including their ability to form $PrP^{Sc}$ (Chesebro et al., 2005; Dear et al., 2007).

$PrP^C$ is expressed ubiquitously expressed throughout the body but is present at higher levels in cells of the central nervous system, particularly neurons, as well as cells in the spleen and heart (Castle et al., 2017). Mature $PrP^C$ has two N-linked glycosylation sites and is found in di-glycosylated, mono-glycosylated, and un-glycosylated forms (Haraguchi et al., 1989). In addition, $PrP^C$ contains a glycophosphatidylinositol (GPI) membrane anchor that tethers it to the cell surface (Puig et al., 2019). $PrP^C$ can also be cleaved by cellular proteases at three different sites, although the purpose of these cleavages is unknown (Linsenmeier et al., 2017). Since $PrP^{Sc}$ is derived from host $PrP^C$, its post-translational modifications may also be present in $PrP^{Sc}$. The amount of each glycoform (Tuzi et al., 2008), the sialylation pattern (Katorcha et al., 2015), and truncation state of $PrP^{Sc}$ (Frankenfield et al., 2005; Notari et al., 2008) can vary between prion strains and may directly influence the properties of $PrP^{Sc}$ (Collinge et al., 1996) and the pathology of prion disease (Nicot et al., 2010). Experimental modification of the properties of $PrP^C$ through truncation (Flechsig et al., 2000), removal of the GPI anchor (Chesebro et al., 2005), or removal of the glycosylation sites (Tuzi et al., 2008) can change how $PrP^{Sc}$ deposits *in vivo*, as well as other aspects of prion pathogenesis, without necessarily leading to new prion strains. Thus, if the prevalence of different post-translational modifications varies between cells, the host $PrP^C$ molecule may help to determine the phenotypes of different prion strains, suggesting that prion disease may be influenced not only by $PrP^{Sc}$ conformation but also by the host cell type.

In contrast to $PrP^C$, $PrP^{Sc}$ is found in a limited number of cell types, primarily neurons in the brain and follicular dendritic cells in the spleen (Beekes et al., 2007). The intracellular concentration of $PrP^{Sc}$ throughout the body of an infected host varies with cell type and prion strain (Beekes et al., 2007; Parchi et al., 1996; Parchi et al., 1999) and the properties of $PrP^{Sc}$, such as sialyation pattern, vary between originating cell types (Srivastava et al., 2015), suggesting that the properties of different cell types may play a role in prion

pathology. For example, $PrP^{Sc}$ produced at high titer in muscle tissue or with high amounts of sialyation in the spleen may migrate through blood or lymphatic fluids to other organs including the brain, potentially contributing to the rapid degeneration of the brain during late-stage prion disease (Beekes et al., 2007; Bosque et al., 2002; Srivastava et al., 2015). Although it has been historically difficult to identify cell lines that are susceptible to prions (Piccardo et al., 2011), the eventual development of multiple *in vitro* cell culture models of prion infection has allowed the underlying mechanisms that enable infection of a cell, as well as other aspects of prion infection, to be studied in more detail. This review will explore how cell line models have furthered our understanding of prion biology and clarified *in vivo* observations regarding the role of sequence variation in the prion protein gene (PRNP) on both prion disease pathology and species barriers to prion disease. In addition, this review will focus on how cell culture models have provided new insights into the establishment and persistence of prion infection, in particular the ways in which different prion strains interact with and potentially infect cells. While many mysteries still persist, such as why some prion strains cannot persistently infect cells *in vitro* or how the structure of a prion strain dictates how it interacts with cellular machinery, it's possible that the development of new *in vitro* cell systems will eventually allow us to map the molecular interactions between $PrP^{Sc}$ and the cell, potentially providing novel targets for drug development.

## From *in vivo* to *in vitro*: dissecting how genetic variation influences prion disease

Certain mutations in human PRNP lead to the spontaneous formation of $PrP^{Sc}$ and the development of human prion diseases such as Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Schienker (GSS) disease (Gambetti et al., 2003). However, only 10 % of all CJD cases arise from heritable mutations that reproducibly lead to $PrP^{Sc}$ formation, which are referred to as familial CJD (fCJD). About 90 % of all CJD cases arise spontaneously and are referred to as sporadic CJD (sCJD). Less than 1 % of human CJD cases are acquired through contact with prion contaminated materials such as prion-contaminated medical equipment or tissues, which results in iatrogenic CJD (iCJD). Exposure to BSE contaminated materials results in variant CJD (vCJD) (Weinstein et al., 2001), a so far unique instance where a prion disease has crossed species barriers to cause disease in humans. While the most common type of prion disease in humans is sCJD, iCJD and vCJD can provide clearer information about human prion pathogenesis, especially with regard to disease incubation times and prion spread. This is in part because the source of infection and exposure can often be determined, especially in the case of iCJD, and in part because the route of infection is known. Polymorphisms in human PRNP can both alter an individual's susceptibility to sCJD (Collinge, 2005; Sangeetham et al., 2021) and fCJD (Monari et al., 1994) (Parchi et al., 1996). For example, the D178N mutation leads to a heritable form of prion disease but the type of disease depends upon a polymorphism at codon 129, with the presence of methionine at codon 129 leading to the development of fatal familial insomnia (FFI) and the presence of valine leading to development of fCJD (Monari et al., 1994). D178N $PrP^{Sc}$ can also have different sizes after proteinase K (PK) treatment depending on the combination of polymorphisms at codon 129, indicating that genetic polymorphism in PRNP can also influence the structure and properties of $PrP^{Sc}$ (Monari et al., 1994; Parchi et al., 1996). In this area of prion research, *in vivo* work has been instrumental in identifying the combination of genetic backgrounds and strains that allow

for prion disease development and the characterization of their unique pathologies. However, the properties of different $PrP^{Sc}$ aggregates, and how they may contribute to the underlying biochemical and molecular interactions that drive disease pathology, can be examined under more controlled conditions using *in vitro* cell line models for prion infection.

**Properties of genetic variants of $PrP^{C}$ that lead to spontaneous formation prion disease—**Several transgenic mouse lines have been developed in recent years that appear to be promising models for the study of spontaneous prion disease formation. For example, mouse models have been generated by replacement of mouse PRNP with either bovine PRNP bearing a leucine mutation at codon 113, which models the human GSS mutation P102L (Torres et al., 2013), or bank vole PRNP bearing a lysine mutation at codon 200, which models the E200K mutation associated familial CJD (Watts et al., 2016). To date, however, no *in vitro* cell culture model has been successfully developed for the study of spontaneous $PrP^{Sc}$ formation and prion infectivity. Mutations associated with fCJD were shown to lead to increased aggregation and protease resistance in $PrP^{C}$, though none led to the spontaneous formation of fully-protease resistant and infectious $PrP^{Sc}$ (Lehmann et al., 1996; Priola et al., 1998). For instance, cerebral organoid cultures derived from patients carrying the E200K mutation do not spontaneously form $PrP^{Sc}$ (Foliaki et al., 2020) despite the E200K mutation in PrP driving $PrP^{Sc}$ formation *in vivo*. Interestingly prion strains that are unable to infect cells *in vitro* are still able to convert $PrP^{C}$ into $PrP^{Sc}$ upon initial cellular interaction (Vorberg, Raines, & Priola, 2004), possibly indicating that the intercellular spaces found *in vivo* may promote the persistence of certain prion strains by allowing for the accumulation of $PrP^{Sc}$ outside of the cell. While it's unclear why aggregation prone mutant $PrP^{C}$ does not spontaneously form $PrP^{Sc}$ in cells *in vitro*, it may be that other cellular deficits, perhaps in the proteostasis machinery that can occur with natural cell aging, are required for the formation of $PrP^{Sc}$.

Despite the inability of *in vitro* cell culture models to spontaneously form $PrP^{Sc}$, they have still proven useful in dissecting the molecular biology of $PrP^{Sc}$ bearing the mutations that drive spontaneous $PrP^{Sc}$ formation *in vivo*. For example, brain homogenates from mice infected with the M1000 mouse adapted GSS strain were used to infect rabbit kidney (RK-13) cells expressing mouse $PrP^{C}$ with the GSS causing mutations G114V and A117V (positions 113 and 116, respectively, in mouse $PrP^{C}$). The cells produced aggregates of $PrP^{Sc}$ that were both smaller and less PK resistant than $PrP^{Sc}$ produced by RK-13 cells expressing wild-type mouse $PrP^{C}$ (Coleman et al., 2014). Despite the smaller aggregate size and lower resistance to PK, $PrP^{Sc}$ from the RK-13 cells expressing mutant $PrP^{C}$ was more infectious when inoculated into mice than $PrP^{Sc}$ from RK-13 cells expressing wild-type $PrP^{C}$ (Coleman et al., 2014). The increased infectivity of G113V and A116V $PrP^{Sc}$ aggregates may be the result of their smaller size as smaller prion aggregates have been found to be more infectious and possess a higher capacity for both prion conversion and neuroinvasion (Bett et al., 2017; Kim et al., 2012; Silveira et al., 2005). These studies illustrate how *in vitro* and *in vivo* data combined can contribute to a better understanding of sporadic prion disease, even when *de novo* infectious prion formation is difficult to observe.

The spontaneous transformation of either wild-type or mutant $PrP^{C}$ into $PrP^{Sc}$ is poorly understood, but it is believed that a critical first step may be the failure of cellular systems

to clear misfolded and aggregated prion protein (Thody et al., 2018). In addition, mutations in PRNP that are associated with the formation of infectious prions *in vivo* are known to alter the properties of $PrP^{C}$ in ways that may lead to the spontaneous formation of infectious prions. For example, in M17 human neuroblastoma cells, un-glycosylated $PrP^{C}$ bearing the Q217R, E200K, and D178N mutations displays aberrant transport to the cell surface. This appears to be the result of conformational instability in the $PrP^{C}$ molecules bearing these mutations driving misfolding and aggregation of prion protein in the absence of glycosylation (Capellari et al., 2000; Petersen et al., 1996; Singh et al., 1997). However, the misfolded and un-glycosylated prion protein that results from these mutations may only contribute to the formation of infectious prions if it is trafficked to the cell surface, as intracellular un-glycosylated $PrP^{C}$, formed via mutations that remove glycosylation, was not found to support prion infection *in vitro* (Salamat et al., 2011). Fully glycosylated forms of $PrP^{C}$ bearing the Q217R, E200K, and D178N mutations that are properly trafficked to the surface of cells may also be conformationally unstable and able to misfold and spontaneously form $PrP^{Sc}$. Residues 217, 200, and 178 reside in the C-terminal region of $PrP^{C}$ which makes up the alpha-helical core of $PrP^{C}$, and the amino acids at these residues either participate in potentially critical ionic interactions across $PrP^{C}$ or reside next to amino acids that do (Pastore et al., 2007). This may explain why Q217R, E200K, D178N, and other mutations in this region (Mead, 2006) potentially destabilize the structure of $PrP^{C}$, promoting aggregation and the eventual conversion into infectious prions.

Mutations in $PrP^{C}$ that result in C-terminal truncation, such as those that terminate $PrP^{C}$ at amino acid residues 226 (Race et al., 2018), 145 (Ghetti et al., 1996; Kitamoto et al., 1993), and 163 (Revesz et al., 2009), also result in spontaneous formation of $PrP^{Sc}$ in humans but with pathological features, such as perivascular amyloid formation, that are distinct from those associated with $PrP^{Sc}$ formed from full length $PrP^{C}$. Similarly, prion infection of transgenic mice expressing mouse $PrP^{C}$ with a mutation to prevent the attachment of the GPI anchor leads to the development of large, extracellular, perivascular deposits of amyloid and a prolonged disease course when compared to prion infected mice expressing GPI anchored $PrP^{C}$, where $PrP^{Sc}$ was found in non-amyloid deposits and disease incubation times were much shorter (Chesebro et al., 2005). $PrP^{Sc}$ without an anchor also appears to be more infectious and less affected by the species barrier between mice and humans (Race et al., 2015). Studies *in vitro* have shown that neural cells expressing anchorless $PrP^{C}$ cannot be persistently infected with prions unless they also express wild-type, anchored $PrP^{C}$ (McNally et al., 2009; Priola et al., 2009), suggesting that $PrP^{Sc}$ infection in mice expressing $PrP^{C}$ without the GPI anchor is mostly extracellular. Overall, the combined *in vitro* and *in vivo* data suggest that the GPI anchor in $PrP^{C}$ is needed to infect a cell and can determine the localization and type of $PrP^{Sc}$ deposited, as well as influence disease incubation times and the transmission of prions across species.

**Modeling prion species barriers *in vitro*—**In a manner analogous to how mutations in PrP can alter the susceptibility of an organism to prion infection, genetic variation between species can form a ‘species barrier’ that hinders prion transmission between species. Thus, effective transmission of prions between species is often dependent upon the sequence of $PrP^{C}$ in the species infected and the species from which $PrP^{Sc}$ originates (Priola et al., 1995;

Scott et al., 1993). White-tail deer and humans share 90% PRNP sequence identity and yet that small sequence difference appears to prevent the transmission of chronic wasting disease (CWD) from deer to humans (Waddell et al., 2018). However, the amount of sequence similarity between PRNP from different species is not the critical determining factor for transmission between species. Bovines and humans share only 86% PRNP sequence identity yet the prion strain that causes BSE has been transmitted to humans, likely through the consumption of BSE contaminated, bovine derived material (Will et al., 1999). While the species barrier between humans and certain animals can protect human populations from prion diseases in specific animal populations, species barriers also pose a problem for the study of human prions as commonly used animal models can be resistant to human prion strains. This barrier was largely overcome with the discovery that transgenic mice expressing $PrP^{C}$ with the same amino acid sequence as $PrP^{Sc}$ were susceptible to infection with prions from another species (Prusiner et al., 1990). With regard to other species of mammals, there is a strong species barrier between mice and hamsters (Prusiner et al., 1990) while rabbits and dogs appear to be resistant to prion infection. However, this resistance is not absolute as transgenic mice expressing rabbit $PrP^{C}$, but not mouse $PrP^{C}$, were susceptible to BSE and mouse prions (Vidal et al., 2015), indicating that non-prion factors specific to rabbits may contribute to their resistance to prion disease. By contrast, bank voles are infectable with prions from a wide range of species, earning them the title ‘universal prion acceptor’ (Watts et al., 2014).

*In vitro*, many of these *in vivo* species barriers have been studied using cell lines expressing species-specific and/or chimeric $PrP^{C}$ and determining which $PrP^{C}$ molecules are able to convert to $PrP^{Sc}$ when exposed to prions from different species. This has allowed the mapping of key amino acid residues involved in the species-specific formation of $PrP^{Sc}$ and expanded our understanding of how infectious prions transmit between species. (Priola, 2013). While non-prion factors may contribute to species barriers to prion disease, the majority of work using *in vitro* systems has focused on the influence of the $PrP^{C}$ amino acid sequence on cross-species transmission of prions. For example, *in vitro* prion infection of canine kidney cells as well as *in vitro* prion amplification assays using canine PrP showed no signs of $PrP^{Sc}$ formation when exposed to mouse, human, cervid, and sheep prions (Fernández-Borges et al., 2017; Polymenidou et al., 2008), suggesting that the dog prion protein was highly resistant to conversion by $PrP^{Sc}$. A mutation replacing asparagine at codon 163 with an aspartic acid was determined to be responsible for the resistance of dog $PrP^{C}$ to conversion to $PrP^{Sc}$ and the correlating mutation in mouse $PrP^{C}$ also inhibited prion infection (Fernández-Borges et al., 2017; Vidal et al., 2020). While the N163D mutation in dogs creates a barrier to infection by all prion strains that have been tested, single codon mutations in PRNP can create a barrier to infection by specific strains from specific species (Priola et al., 1995). For example, replacing an isoleucine in mouse $PrP^{C}$ at residue 138 with a methionine found at the corresponding residue of the hamster $PrP^{C}$ sequence was sufficient to prevent $PrP^{Sc}$ formation in mouse prion infected mouse neuroblastoma (N2A) cells (Priola et al., 1995). Similar studies between different species have enabled the mapping of specific regions of $PrP^{C}$, in many cases down to a single amino acid residue, that are responsible for the species-specific formation of $PrP^{Sc}$ (Priola, 2013). For example, a proline to leucine mutation in mouse PRNP at codon 101 can significantly

slow the progression of vCJD in mice and valine at codon 129 in human PRNP protected against the transmission of bovine spongiform encephalopathy (BSE) while the methionine polymorphism at codon 129 did not (Barron et al., 2001; Collinge, 2005; Parchi et al., 1996; Wadsworth et al., 2004). These studies have shown that the most important regions and amino acids for interspecies prion transmission differ depending upon the species involved in transmission and whether the polymorphism is present in $PrP^{Sc}$ or the $PrP^{C}$ molecule being converted to $PrP^{Sc}$. This suggests that conformational differences associated with these amino acid variations are the driving force behind the species-specific formation of $PrP^{Sc}$.

## Factors that mediate cellular association and uptake of $PrP^{Sc}$ and their role in disease

The interaction of $PrP^{Sc}$ with cellular membrane and its subsequent internalization by cells are important steps in prion biology that are mediated by various biological and biochemical factors like the GPI anchor (McNally et al., 2009) and membrane bound biomolecules (Gauczynski et al., 2006; Hijazi et al., 2005; Horonchik et al., 2005; Morel et al., 2005). $PrP^{C}$ primarily localizes to membranes via the GPI anchor which has been found to both facilitate persistent infection of *in vitro* cell lines and to alter the pathology of prion disease *in vivo* (Chesebro et al., 2005; McNally et al., 2009; Priola et al., 2009). However, other membrane biomolecules, such as glycosaminoglycans (GAGs), also participate in membrane association, guide the cellular uptake, and influence the properties of $PrP^{Sc}$ in a strain and cell type dependent manner (Hijazi et al., 2005; Horonchik et al., 2005; Imamura et al., 2016; Mayer-Sonnenfeld et al., 2008; Vieira et al., 2014; Wong et al., 2001). These factors were found to be so critical for the propagation of $PrP^{Sc}$ that early drug treatments for prion disease were based around mimetics for these biomolecules (Adjou et al., 2003). Continued study of these factors may help us to better understand how $PrP^{Sc}$ relies on different cellular components for propagation, fostering the advancement of mimetic drugs to fight prion disease.

**The influence of GPI anchoring on the persistence, propagation, and spread of $PrP^{Sc}$—**The C-terminal GPI anchor that tethers $PrP^{C}$ to cell membranes is also present in aggregates of $PrP^{Sc}$ (Stahl et al., 1990) and may facilitate attachment of $PrP^{Sc}$ to membranes. Enzymes like phospholipase C normally cleave the GPI anchor from $PrP^{C}$ but, in the prion strains tested thus far, the GPI anchor of $PrP^{Sc}$ is protected from cleavage by enzymes like phospholipase C (Stahl et al., 1990) which may allow $PrP^{Sc}$ to stay attached to membrane for long periods of time. However, GPI mediated cell surface association is not absolutely required for prion infection *in vivo* as mice bearing mutations in PRNP that remove the GPI anchor can still sustain prion infection (Chesebro et al., 2010; Chesebro et al., 2005). Enzymatically removing the GPI anchor from $PrP^{Sc}$ does not significantly alter its conversion capability or infectivity (Lewis et al., 2006), and anchorless $PrP^{Sc}$ derived from prion infected mice expressing anchorless $PrP^{C}$ is able to infect cell lines expressing GPI-anchored mouse $PrP^{C}$ (McNally et al., 2009). However, prion strains that are normally able to persistently infect cell lines *in vitro* fail to do so when the cells express anchorless $PrP^{C}$ alone (McNally et al., 2009; Priola et al., 2009). The failure of prions to infect cells expressing only anchorless $PrP^{C}$ can be corrected by co-expression of anchored $PrP^{C}$,

which results in persistent infection and formation of both anchored and anchorless $PrP^{Sc}$ (McNally et al., 2009; Priola et al., 2009).

These data suggest that the GPI anchor may influence the localization of $PrP^{Sc}$ and that the presence of an anchor on $PrP^{C}$ may facilitate the spread of infectious prions from cell to cell. This latter prediction was supported through subsequent studies *in vivo* which showed that anchored $PrP^{Sc}$ propagated along neurons (Klingeborn et al., 2011) while anchorless $PrP^{Sc}$ propagated along interstitial fluid drainage systems (Rangel et al., 2014). While $PrP^{C}$ is initially produced with a GPI anchor, $PrP^{C}$ *in vivo* exists as both anchored and anchorless $PrP^{C}$ as enzymes like phospholipase C and the ADAM 10 protease both remove its GPI anchor (Altmeppen et al., 2015; Stahl et al., 1990). Interestingly, when ADAM10 activity was inhibited *in vivo* reducing the amount of $PrP^{C}$ with cleaved GPI anchors, the spread of $PrP^{Sc}$ was hindered (Altmeppen et al., 2015). These data suggest that the spread of $PrP^{Sc}$ in the brain may also be facilitated by the presence of $PrP^{C}$ with enzymatically removed GPI anchors. In this case, anchorless $PrP^{C}$ would not be restricted to the cell surface and would be able to move more freely, likely via the interstitial fluid (Rangel et al., 2014), thus facilitating the spread of $PrP^{Sc}$ throughout the brain (Chesebro et al., 2005).

**The role of GAGs in prion biology—**In animal tissue, GAGs are ubiquitously present linear polysaccharides that associate with membranes, attach to proteins to form proteoglycans, and regulate interactions between proteins. (Horonchik et al., 2005; Li et al., 2016; Shyng et al., 1995; Simon Davis et al., 2013; Warner et al., 2002). GAGs also appear to play an important role in prion biology as binding of the GAG heparan sulfate to $PrP^{C}$ stimulates the internalization of $PrP^{C}$ by cultured cells (Gabizon et al., 1993; Shyng et al., 1995) and heparan sulfate is associated with amyloid plaques of $PrP^{Sc}$ from prion infected mice (McBride et al., 1998). In addition, under-sulfation of heparan sulfate, which resulted from *Papss2* gene silencing, was found to increase the deposition of $PrP^{C}$ in the extracellular matrix and facilitate the propagation of $PrP^{Sc}$ (Marbiah et al., 2014). Incorporation of heparan sulfate into $PrP^{Sc}$ aggregates likely happens during the aggregation process as it was found to promote prion replication and infectivity (Imamura et al., 2016; Shaked et al., 2001; Wong et al., 2001), and GAG mimetic compounds can slow the progression of prion disease *in vivo* (Ehlers et al., 1984; Farquhar et al., 1986). While heparan sulfate facilitates endocytosis for a wide range of biomolecules (Christianson et al., 2014) and has been observed facilitating internalization of $PrP^{Sc}$ (Hijazi et al., 2005; Horonchik et al., 2005), it is not always required for $PrP^{Sc}$ uptake by cells (Paquet et al., 2007; Wolf et al., 2015). One possible explanation for this discrepancy is that in studies where GAGs promoted $PrP^{Sc}$ uptake, purification of the $PrP^{Sc}$ used may have removed potential cofactors that promote an association between $PrP^{Sc}$, GAGS, and the cellular membrane. Alternatively, in studies that did not observe GAG mediated cellular uptake of $PrP^{Sc}$, GAG depleted cells were inoculated with $PrP^{Sc}$ infected brain homogenate which may have contained enough brain-derived GAGs, or other cofactors, to facilitate cellular uptake.

Regardless of whether GAGs are critical for prion uptake or whether GAG independent uptake mechanisms exist, GAGs such as heparan sulfate clearly impact prion disease progression *in vivo* and can influence prion infection of cells in a strain dependent manner (Das et al., 2020; Das et al., 2017; Wolf et al., 2015). The prion protein has three binding

sites for heparan sulfate that can be found at amino acids 23 to 52, 53 to 93, and 110 to 128 in human $PrP^{C}$ (Warner et al., 2002). These sites may play different roles in the propagation of different prion strains as conformational variations between prion strains may influence how well heparan sulfate associates with these sites. This idea is supported by studies in C57BL/6 mice expressing $PrP^{C}$ with mutated heparan sulfate binding sites that were infected with either the 22L or RML prion strains, both of which are mouse adapted sheep scrapie strains that have similar incubations times in C57BL/6 mice (Das et al., 2020; Das et al., 2017). Mutations in $PrP^{C}$ that disrupted heparan sulfate binding to the N-terminal amino acids from 25 to 90 had different impacts on disease progression in the two strains, with RML infected mice having longer incubation times than 22L infected mice (Das et al., 2020; Das et al., 2017). Mutations in the heparan sulfate binding sites of $PrP^{C}$ may lengthen the incubation times of the RML strain by reducing the amount of RML $PrP^{Sc}$ maintained by cells. This possible explanation is supported by *in vitro* studies in L929 mouse fibroblast cells expressing wild-type mouse $PrP^{C}$, which can be infected by both the RML and 22L prion strains (Vorberg, Raines, Story, et al., 2004). A reduction in sulfated GAG levels, including heparan sulfate, through GAG mimetic dextran sulfate 500 (DS-500) and sodium chlorate treatment resulted in a greater decrease in cellular RML $PrP^{Sc}$ concentrations when compared to 22L (Wolf et al., 2015). Heparan sulfate has also been found to promote the uptake of $PrP^{Sc}$ and it may be that the RML strain is more dependent upon it for internalization when compared to the 22L strain. Alternatively, it is possible that heparan sulfate is more important for the structural stability of the RML strain than the 22L strain. $PrP^{Sc}$ from these strains varies in its structural stability, with $PrP^{Sc}$ from the 22L strain being thermally more stable than $PrP^{Sc}$ from the RML strain (Marín-Moreno et al., 2019). Heparan sulfate has been found to stabilize the structure of RML prions which may explain why, when heparan sulfate is forcibly accumulated in cells, the ability of the lysosome to clear RML $PrP^{Sc}$ is delayed (Mayer-Sonnenfeld et al., 2008; Vieira et al., 2014). The strain specific interaction of GAGs with $PrP^{Sc}$ may also help to explain the pattern of $PrP^{Sc}$ deposition in the host. The concentration and characteristics of heparan sulfate and other GAGS varies between tissues (Clark et al., 2011; Warda et al., 2006) and $PrP^{Sc}$ from prion strains like RML may be more stable and may accumulate to higher concentrations in cells and areas of the tissue with higher heparan sulfate concentrations. While variation in GAG distribution and characteristics between cell lines may promote strain-specific differences in $PrP^{Sc}$ accumulation in different cells, variability in the way different cell types treat different prion strains, such as differences in the endocytic uptake route of $PrP^{Sc}$, may also contribute (Fehlinger et al., 2017).

**Potential protein cofactors for cell association and uptake of $PrP^{Sc}$—**Another way in which GAGs may influence PrP uptake is by altering interactions between clathrin coated pits and prions (Figure 1A). Clathrin-mediated uptake of $PrP^{C}$ requires interactions with both heparan sulfate and the laminin receptor protein (LRP) (Gauczynski et al., 2001; Hundt et al., 2001), and there is evidence that LRP may facilitate internalization of $PrP^{Sc}$ with some cell lines depending upon LRP for prion uptake more than others (Gauczynski et al., 2006; Morel et al., 2005). Two cell lines with seemingly different dependencies on LRP for uptake of $PrP^{Sc}$ are Baby Hamster Kidney (BHK) cells and the human colon adenocarcinoma cell line Caco-2TC7. Inhibition of GAGs in BHK cells inhibited $PrP^{Sc}$

uptake independent of LRP concentration, while antibody mediated inhibition of LRP in Caco-2TC7 cells inhibited $PrP^{Sc}$ uptake (Gauczynski et al., 2006; Morel et al., 2005). This may mean that in some cell types LRP is more critical for $PrP^{Sc}$ uptake while in other cell lines GAGs are more critical. Alternatively, the low-density lipoprotein receptor related protein (LRP1) may also facilitate uptake of $PrP^{Sc}$ as inhibition of LRP1 in both primary neuron and hippocampal cell cultures prevented uptake of $PrP^{Sc}$ from the prion strain ME7, which is a mouse adapted sheep scrapie strain (Jen et al., 2010). While only the single study shows that LRP1 contributes to $PrP^{Sc}$ uptake, numerous studies have shown that LRP1 may be involved in Alzheimer's disease as LRP1 regulates uptake of Aβ and tau aggregates (Lane-Donovan et al., 2014; Rauch et al., 2020; Zhang et al., 2020), demonstrating that it can facilitate the uptake of a multiple non-native aggregates. Interestingly, $PrP^{C}$ is also involved in tau aggregate uptake, which can inhibit the accumulation of $PrP^{Sc}$ (De Cecco et al., 2020). This may indicate that membrane proteins, other than LRP1, that are critical for membrane interaction and cellular uptake of Aβ and tau aggregates in Alzheimer's disease may also be important for membrane interaction and cellular uptake of $PrP^{Sc}$.

## Endocytosis pathways and endosomal trafficking of $PrP^{Sc}$

Determining which cell types can be infected by prions and why is critical for understanding how prion infection is established as well as how prions replicate and spread. Upon initial interaction with the cell, $PrP^{C}$ is rapidly converted into $PrP^{Sc}$ regardless of the prion strain or its ability to persistently infect the cell with which it is interacting (Goold et al., 2011; Vorberg, Raines, & Priola, 2004). Prion conversion can happen across the cell surface (Goold et al., 2011) but $PrP^{C}$ can also accumulate within cell surface features, such as lipid rafts, which may be critical sites of prion conversion (Hooper, 2011; Lewis et al., 2011; Vey et al., 1996). In vitro, the amount of $PrP^{Sc}$ taken up by cells is also independent of strain and whether a cell line can be infected by a specific prion strain (Greil et al., 2008). However, the rate at which $PrP^{Sc}$ is degraded by the cell is strain dependent (Choi et al., 2013; Shoup et al., 2021) and may be determined by the stability of the $PrP^{Sc}$ aggregate (Shoup et al., 2021). This suggests that the ability of a strain to infect a cell may be in part the result of cellular factors that control how rapidly $PrP^{Sc}$ is trafficked to the lysosome for degradation versus how well newly formed $PrP^{Sc}$ is trafficked away from the lysosome. How aggregates of $PrP^{Sc}$ from different strains interact with cells may influence the rate at which they are trafficked, as fluorescent $PrP^{Sc}$ aggregates that were found to form strings on the cell surface were taken up very slowly (Rouvinski et al., 2014). Thus, the route by which $PrP^{Sc}$ is endocytosed as well as how $PrP^{Sc}$ is trafficked through the endocytic pathway are potentially important factors that are likely to vary with cell type, as they do with the uptake of other molecules (Douglas et al., 2008), and may help determine whether a particular cell can be infected by a specific strain of prion (Fehlinger et al., 2017).

**Routes of endocytosis for $PrP^{Sc}$ and $PrP^{C}$—**Endocytosis is critical for maintaining homeostasis in multicellular organisms and thus can be difficult to manipulate and study in detail using *in vivo* models. As such, *in vitro* cell line models have been particularly useful in furthering our understanding of how cells internalize and traffic $PrP^{C}$ and $PrP^{Sc}$. How cells internalize $PrP^{C}$ is an important consideration in prion disease as endocytic vesicles that contain $PrP^{C}$ may be central sites of prion conversion if cells take up $PrP^{Sc}$ via the same

route that $PrP^C$ is taken up. Cell surface associated $PrP^C$ is predominately internalized via clathrin-coated pits and caveolae before trafficking to the early endosome (Figure 1A,B, and D) with uptake of $PrP^C$ by these mechanisms being modulated by association of $PrP^C$ with lipid rafts and metal ions (Hooper et al., 2008; Peters et al., 2003; Sarnataro et al., 2009). The exact uptake pathway for $PrP^C$ can also be cell type dependent. L929 cells, a murine fibroblast cell line, predominately internalize $PrP^C$ via clathrin-coated pits as opposed to utilizing caveolae (Fehlinger et al., 2017). While $PrP^C$ uptake in L929 cells was independent of caveolin-mediated endocytosis, knocking down caveolin led to cell surface accumulation of $PrP^C$, indicating that caveolin may play a role in $PrP^C$ uptake even if it is not required for internalization (Fehlinger et al., 2017). Since GPI anchored proteins like $PrP^C$ can accumulate on lipid rafts, inhibition of caveolin-mediated uptake may cause $PrP^C$ to remain on the cell surface with no way to leave lipid rafts or be internalized, which would explain why $PrP^C$ accumulates on the surface of cells when caveolin-mediated uptake is inhibited. Thus, even though $PrP^C$ is predominately taken up by clathrin-mediated endocytosis in L929 cells, $PrP^C$ that becomes trapped in lipid rafts is likely only taken up by caveolae mediated endocytosis. As previous studies have shown that $PrP^C$ and $PrP^{Sc}$ co-localize to lipid rafts (Hooper, 2011; Lewis et al., 2011; Vey et al., 1996) it may be that caveolae-mediated uptake plays a role in persistent infection of L929 cells. Lipid rafts may also facilitate the spread of $PrP^{Sc}$ between cells as anchorless $PrP^C$ has also been found to associate with membranes via lipid rafts and is capable of being converted by $PrP^{Sc}$ while attached to lipid rafts *in vitro* (Baron et al., 2003). In addition, lipid rafts may only function as sites of prion conversion on the surface of cells, or in early endosomal compartments, as *in vitro* prion conversion assays on lipid rafts showed that conversion of GPI anchored $PrP^C$ was optimal between a pH of 6 to 7 (Baron et al., 2002).

Cellular uptake of $PrP^{Sc}$ via macropinocytosis was observed in N2a cells (Wadia et al., 2008), and clathrin-mediated endocytosis may participate in uptake of $PrP^{Sc}$ as laminin receptors were found to be involved in $PrP^{Sc}$ uptake in Caco-2/TC7 cells (Morel et al., 2005). Caveolae may also either directly internalize $PrP^{Sc}$ or assist $PrP^{Sc}$ uptake through other endocytic pathways. $PrP^{Sc}$ has been found associated with caveolae on the surface of N2a cells, and both inhibition of caveolin in N2a cells and knock down of caveolin in L929 cells reduced the internal accumulation of $PrP^{Sc}$ in chronically infected cells (Fehlinger et al., 2017; Marella et al., 2002; Vey et al., 1996). One possible explanation for how knocking down or inhibiting caveolin can reduce intracellular accumulation of $PrP^{Sc}$ without interfering with cellular internalization may be that inhibition of one endocytic route often causes upregulation of other endocytic routes to compensate for the lost endocytic pathway (Fehlinger et al., 2017). Some endocytic routes may provide more favorable cellular microenvironments for establishing persistent cellular infection. Evidence supporting this was derived from studies in L929 cells which showed that inhibition of clathrin-mediated endocytosis increased uptake of FITC-dextran via macropinocytosis as well as $PrP^{Sc}$ (Figure 1C) (Fehlinger et al., 2017). While these data, in conjunction with data from primary neuronal culture (Bett et al., 2017), suggests that uptake via macropinocytosis may facilitate persistent infection of cells by 22L prions, the level of RML $PrP^{Sc}$ in chronically infected L929 cells decreases with a reduction in clathrin mediated endocytosis activity (Fehlinger

et al., 2017). Since both RML and 22L prions can infect L929 cells, these data suggest that they may do so most efficiently via different endocytic routes.

**Variations in vesicle microenvironment and their influence in prion biology—** The microenvironment of endocytic vesicles and the treatment of the cargo they carry varies between different endocytic routes early in the endocytosis process. Variable treatment of prion protein cargo between different endocytic routes may therefore promote or disrupt prion replication, stability, and aggregation. One example of an endocytic vesicle component that influences prion biology is the lipid raft. Lipid rafts are sites of $PrP^{C}$ localization and potentially prion replication as $PrP^{Sc}$ can co-localize with $PrP^{C}$ in the lipid raft (Hooper, 2011; Lewis et al., 2011; Vey et al., 1996). Lipid rafts are not necessary for clathrin-mediated endocytosis or macropinocytosis, though lipids rafts can be taken up through macropinocytosis (El-Sayed et al., 2013). However, caveolae formation does require lipid rafts, which are drawn into the vesicles during endocytosis and may thus facilitate prion replication if both $PrP^{C}$ and $PrP^{Sc}$ are present (Kiss et al., 2009; Lajoie et al., 2010). While lipid rafts may facilitate prion conversion by bringing $PrP^{C}$ and $PrP^{Sc}$ together (Baron et al., 2003; Baron et al., 2002), other characteristics of endocytic vesicles that may vary during early endocytosis, such as intravesicular pH and ion concentration, (Maxfield, 2014; Sonawane et al., 2002; Tsang et al., 2000) may influence prion conversion by altering prion structure (DeMarco et al., 2007; Nandi et al., 2002; Zanusso et al., 2001). Endocytic vesicles that result from caveolae- and clathrin-mediated endocytosis transition through early, late, and lysosomal vesicles where the general pH of each intracellular compartment is generally 6.5, 5.5, and 4.5 respectively (Hu et al., 2015) (Figure 1D,E, and G). However, the pH of vesicles formed from macropinocytosis can drop to a pH of 5.1 within 10 minutes of vesicle formation and stay at that low pH until the vesicle merges with the lysosome (Tsang et al., 2000) (Figure 1G). The decreasing pH experienced by vesicle cargo at different steps of endocytosis and endosomal trafficking may promote prion conversion as $PrP^{C}$ has been found to misfold at lower pH values which may facilitate conversion into non-native $PrP^{Sc}$ conformations (DeMarco et al., 2007; Thompson et al., 2018). In addition, changes in pH that have been found to alter the stability of $PrP^{Sc}$ in a strain specific manner may also facilitate $PrP^{Sc}$ formation (Zanusso et al., 2001). The potential influence of pH on prion conversion and stability is further compounded by changes in vesicle pH being accompanied by intravesicular increases in anionic salts (Sonawane et al., 2002), which can alter the structural stability of $PrP^{Sc}$ (Concha-Marambio et al., 2014; Nandi et al., 2002). Thus, differences in the chemical environment of vesicles as a direct result of cellular internalization via different endocytic pathways, including how fast the environments change and how long prion cargo is incubated in each microenvironment, may either facilitate or hinder the ability of a particular prion strain to establish a persistent infection by affecting the conversion of $PrP^{C}$ to $PrP^{Sc}$.

The dynamic chemical microenvironment of vesicles traversing the endosomal network from different cellular internalization pathways undoubtedly influences prion biology but morphological variations in vesicles, such as those observed in the macropinosome, may also play a role (Buckley et al., 2017) (Figure 1F). Work in human embryonic kidney (HEK) cells revealed macropinosome vesicles are divided into smaller vesicles through

constriction of portions of the membrane into long tubes (Kerr et al., 2006). These tubulated macropinosome vesicles migrate to early endosomal (Figure 1D) vesicles while the remainder of the un-tubulated vesicle migrates toward the lysosome (Buckley et al., 2017; Kerr et al., 2006). Prion proteins caught in macropinosome tubules may be forced into close proximity, promoting both conversion into and aggregation of $PrP^{Sc}$. Membrane tubulation can also occur in multi-vesicular bodies, also known as the late endosome, (Woodman et al., 2008) and these vesicle compartments have been identified as key sites for prion conversion (Yim et al., 2015). Other intracellular compartments, such as the trans-golgi network or recycling endosome (Figure 1H), also possess unique morphologies or undergo morphological transformations such as tubulation (Rambourg et al., 1979; Van Ijzendoorn, 2006) that may alter aggregated $PrP^{Sc}$ or promote the propagation of $PrP^{Sc}$ trafficked to these compartments (Goold et al., 2013; Yamasaki et al., 2018). Trafficking of $PrP^{Sc}$ between endosomal compartments may influence prion propagation by exposing $PrP^{Sc}$ to new endosomal microenvironments but may also provide a way for newly formed $PrP^{Sc}$ to escape trafficking to the lysosomal as intracellular compartments, such as the recycling endosome, can traffic $PrP^{Sc}$ back to the cells surface (Figure 1H) (Grant et al., 2009). *In vivo*, data consistent with localization of PrPSc to the lysosome has been reported in the brains of human CJD and scrapie infected mice (Ironside et al., 1993; Kovács et al., 2007; Laszlo et al., 1992). $PrP^{Sc}$ has also been found trafficked back to the surface of cells via exosomes (Hartmann et al., 2017) and may have experienced different vesicle microenvironments compared to $PrP^{Sc}$ released from cells via endosomal recycling vesicles. In either case, passage thru cells may alter the properties of $PrP^{Sc}$ which may contribute to the progression of prion disease in other cells in unique ways after release from one cell and re-uptake by another.

**$PrP^{Sc}$ interferes with trafficking of vesicles to the lysosome—**Regardless of the route of endocytosis, *in vitro* most $PrP^{Sc}$ is ultimately trafficked to the lysosome (Caughey et al., 1991) (Figure 1G), a finding consistent with *in vivo* observations suggesting that $PrP^{Sc}$ can be found in lysosomes in the brains of human CJD and scrapie infected mice (Ironside et al., 1993; Kovács et al., 2007; Laszlo et al., 1992). However, $PrP^{Sc}$ may be able to delay trafficking to the lysosome by interfering with the proteins that regulate normal endosomal trafficking, potentially allowing it more time to convert $PrP^{C}$ before it degrades. In N2a cells infected with 22L and RML prions, the amount of membrane bound Rab7, a protein that regulates trafficking to lysosomal vesicles through association with the vesicle membrane, was decreased. This indicated that lysosomal activity was impaired in N2a cells chronically infected with prions (Shim et al., 2016). However, a comparison of global protein degradation rates in N2a cells that were or were not infected with RML prions found that proteasomal degradation rates actually increased during infection and that RML $PrP^{Sc}$ had a half-life 1.7 times greater than $PrP^{C}$ in chronically infected cells (Hutti et al., 2020). This suggests that Rab7 may only fail to associate with vesicles containing $PrP^{Sc}$, thus preventing trafficking of $PrP^{Sc}$ to the lysosome. Rab proteins have been found to co-purify with $PrP^{Sc}$ (Moore et al., 2010), suggesting that interference with the association of Rab7 with membrane vesicles may result from a direct interaction between $PrP^{Sc}$ and Rab proteins. Blocking of the Rab7 association with lysosomal vesicles may also result from interference with other endosomal proteins, such as sortilin, which is a sorting receptor

for endocytosis. In N2a cells, sortilin overexpression reduces the amount of intracellular 22L and RML $PrP^{Sc}$ and under expression increases the internal concentration of both proteins (Uchiyama et al., 2017). Sortilin also appears to direct $PrP^{Sc}$ to lysosomal vesicles (Uchiyama et al., 2017). However, the expression of sortilin is decreased during prion infection, suggesting that while sortilin may play a role in directing $PrP^{Sc}$ to the lysosome, $PrP^{Sc}$ may interfere with sortilin function during prion infection (Uchiyama et al., 2017). Again, disrupting the normal transition of $PrP^{Sc}$ to the lysosome may give it more time to convert $PrP^{C}$ into $PrP^{Sc}$ leading to the accumulation of more $PrP^{Sc}$ and potentially giving newly formed $PrP^{Sc}$ more time to escape vesicles bound for the lysosome.

## Conclusions

*In vitro* studies have proven critical in uncovering the molecular mechanisms by which different prion strains produce distinct disease pathologies *in vivo*. The mechanistic insights gained from *in vitro* studies have furthered our understanding of how different means of cellular interaction, uptake, and intracellular trafficking contribute to prion spread, uptake, degradation, and the ability to persistently infect cells. In addition, *in vitro* studies have clarified how genetic variation leads to transmission barriers between species and effects infectious prion formation. Further development of more complex, three-dimensional cell systems, such as cerebral organoids (Foliaki et al., 2020) may help emulate the extracellular spaces found *in vivo*, providing the proper environment to maintain and study extracellular $PrP^{Sc}$ formation. Prion strains that cannot infect cell lines can still be taken up and degraded by cells, making the processes of cellular uptake, endosomal trafficking, and lysosomal degradation a common denominator in the biology of all prion strains. How prions interfere with or bypass normal trafficking to the lysosome may be exploited for the development of drugs that prevent prions from escaping lysosomal degradation. Taken together, the mechanistic insights into prion biology provided by *in vitro* studies have deepened our understanding of prion biology, providing multiple new avenues for the development of anti-prion therapeutics.

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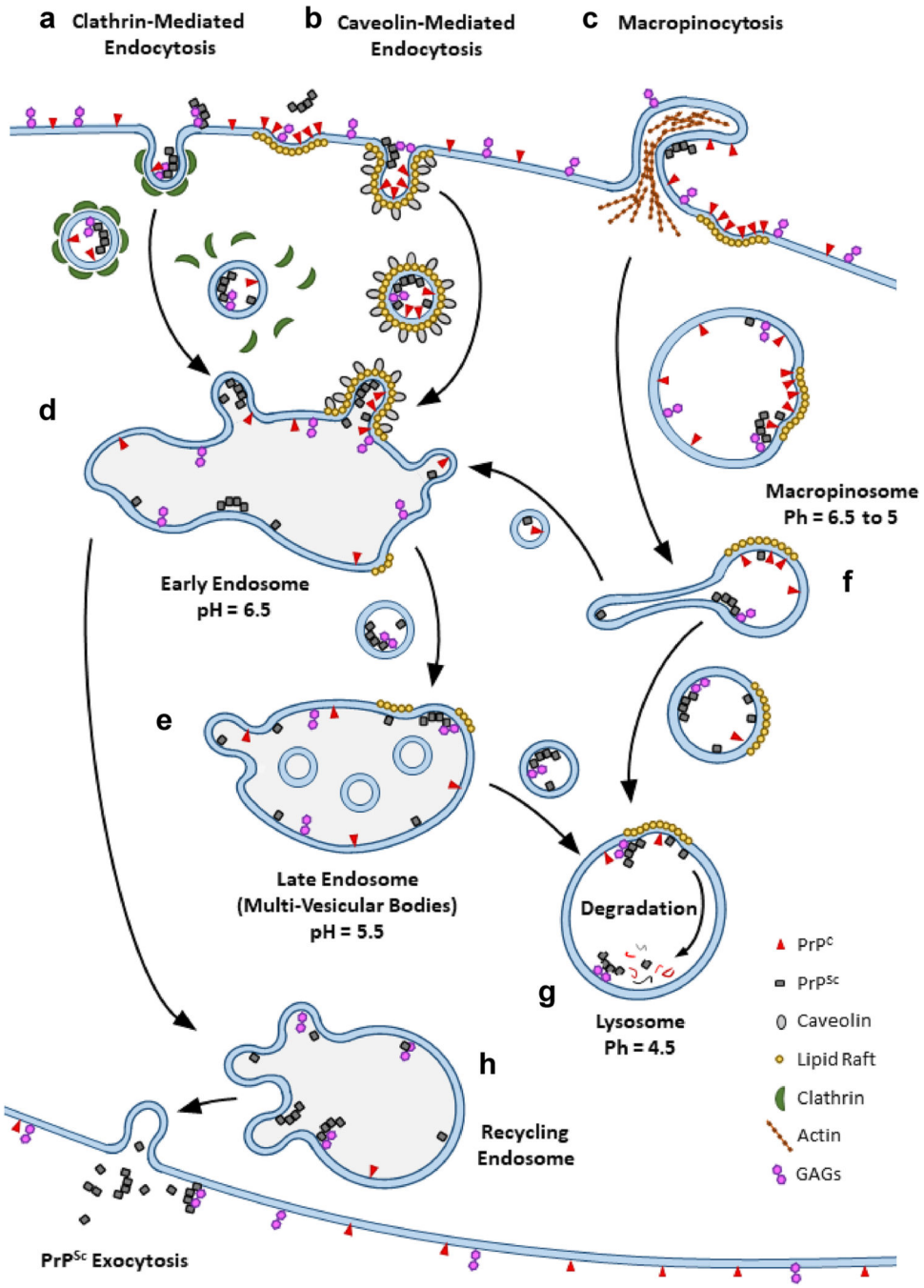


**Figure 1 - Uptake, trafficking, conversion, and degradation of $PrP^{Sc}$ -**

Cellular uptake of $PrP^{Sc}$ through three different routes and subsequent trafficking pathways to the lysosome are shown. $PrP^{Sc}$ (grey squares) may be taken up via (A) clathrin-mediated endocytosis, (B) caveolin-mediated endocytosis, and (C) macropinocytosis alongside $PrP^{C}$. Endosomal vesicles formed via clathrin-mediated and caveolin-mediated endocytosis are typically directed to the (D) early endosomal vesicles before $PrP^{Sc}$, and other vesicle cargo, is trafficked into (E) late endosomal vesicles, also known as multi-vesicular bodies. Vesicles formed via macropinocytosis become a part of the (F) macropinosome where the cargo of

large vesicles can be separated into different smaller vesicles by tubulation and shuttled to early endosomal vesicles. Over time, the remains of tubulated macropinosomal vesicles and the contents of late endosomal vesicles are shuttled to the (G) lysosome for degradation. Contents of the early endosome may also be trafficked to the (H) recycling endosome and then onto the cell surface for expulsion. Molecules such as $PrP^{C}$ (red triangles) and GAGs (purple hexagons) can co-migrate with $PrP^{Sc}$ through different routes of uptake and endosomal trafficking. GAGs promote the uptake, conversion, and the stability of $PrP^{Sc}$ while $PrP^{C}$ can associate with $PrP^{Sc}$ at numerous cellular locations from the cell surface to the pre-lysosomal vesicles. While a significant amount of prion conversion can happen on the surface of cells, conversion of $PrP^{C}$ to $PrP^{Sc}$ has been observed in several pre-lysosomal vesicles (shaded vesicles). GPI anchored $PrP^{C}$ can also accumulate on lipid rafts (gold circles), making lipid rafts potentially important sites of prion conversion at numerous cellular locations.