Functions of the cellular prion protein, the end of Moore's law, and Ockham's razor theory

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ABSTRACT. Since its discovery the cellular prion protein (encoded by the *Prnp* gene) has been associated with a large number of functions. The proposed functions rank from basic cellular processes such as cell cycle and survival to neural functions such as behavior and neuroprotection, following a pattern similar to that of Moore's law for electronics. In addition, particular interest is increasing in the participation of *Prnp* in neurodegeneration. However, in recent years a redefinition of these functions has begun, since examples of previously attributed functions were increasingly re-associated with other proteins. Most of these functions are linked to so-called "*Prnp*-flanking genes" that are close to the genomic locus of *Prnp* and which are present in the genome of some *Prnp* mouse models. In addition, their role in neuroprotection against convulsive insults has been confirmed in recent studies. Lastly, in recent years a large number of models indicating the participation of different domains of the protein in apoptosis have been uncovered. However, after more than 10 years of molecular dissection our view is that the simplest mechanistic model in PrP^C-mediated cell death should be considered, as Ockham's razor theory suggested.

KEYWORDS. Prion, PrP, neurodegeneration

IS THERE PLENTY OF ROOM AT THE BOTTOM OF PRP^C^ ?

"There's Plenty of Room at the Bottom" was a lecture given by physicist Richard Feynman at the American Physical Society meeting at Caltech in December 1959. He was particularly interested in the possibility of increasing computer circuitry especially in ultramicroscopes to achieve higher resolution than the electron microscopes of the time. In fact, Feyman's talk is considered for many researchers the starting point of modern nanotechnology. The talk was republished in the 1990s, 25 years after Gordon Moore, director of research and development at Fairchild Semiconductors and co-founder of IBM, hypothesized in *Electronics* Magazine a doubling every year in the number of components per integrated circuit (later called Moore's Law). In fact, Moore's view of electronics also expanded to molecular biology when Rob Carlson predicted in 2003 a hypothetical increase in DNA sequencing capabilities (measured by cost and performance) with similar doubling to that of Moore's law (the Carlson curve, published by *The Economist*, August 31, 2006). However, Moore foresaw in March, 2015 that the rate of circuit progress would reach saturation: *"I guess I see Moore's law dying here in the next decade or so, but that's not surprising!."* We have the same perception, with the inclusion of new circuits in a finite space reaching saturation point. In fact, this saturation seemed to have started in 2011 (Fig. 1 obtained from https://commons.wikime dia.org/wiki/User:Wgsimon). In addition, the Carlson curve was rendered outdated in 2008 with the development of the new DNA sequencing technologies.

Now, we need to move in this commentary from theoretical electronics to neuroscience, and in particular to prion biology. Numerous manuscripts dealing with PrP^C^ begin with sentences similar to these: "The physiological function of the prion protein is not yet known" or "PrP^C^ plays a key role in the pathogenesis of prion diseases, but its physiologic function remains unclear." This occurs simply as a result of the published descriptions, since we would be astonished with the *"terrific"* number of distinct cellular processes linked to the cellular prion protein (a GPI-anchored protein with a finite molecular space of 231 residues): cell survival and differentiation,[1,2] oxidative stress,[3,4] copper homeostasis,[5,6] cell proliferation[7,8] and cell-cell signaling[9,10] are all fully associated with or participated in by PrP^C^. In fact, most of these functions were deduced or supported by experiments using as experimental model one of first generated *Prnp^o/o^* mice: the Zurich I mouse.[11] Using this mouse, the first studies were directed to clearly determine that *Prnp* expression is mandatory to prion infection and propagation (e.g.[12]). But the few functional alterations initially described in Zurich I were complemented by Edinburgh *Prnp^o/o^* mice, the second model generated around this time.[13] However, since 1992, sequentially published studies have identified a large number of phenotypic effects of the absence of *Prnp* including depressive-like behavior,[14,15] cognitive deficits,[16] peripheral myelin deficits,[17] age-dependent behavioral abnormalities,[18] altered olfaction,[19] altered circadian rhythms,[13] altered associational learning,[20] altered sleep recovery,[21] altered increased susceptibility to oxidative stress,[3] increased excitotoxicity[22-25] and altered neural stem cell proliferation.[1,26] The descriptions of new functions almost followed the slope of the Moore's law but in some cases with controversy (see refs. 27 and 28 for details) (see Appendix for some examples). For a GPI-anchored protein, the proposed list of functions seemed to be at least disproportionate. Relevantly, last year functional descriptions reached a plateau and some functions started to be re-assigned to other proteins after careful re-evaluation of the role of PrP^C^ in these processes. In this respect, one putative explanation is to consider that some of these functions are not directly mediated by PrP^C^ and might depend on extracellular or intracellular partners of the protein. This may be the case for some functions, but while other extracellular interactions and their physiological relevance are well established (for example with adhesion molecules,[29,30] lipoprotein receptors,[31] laminin receptor,[32,33] amyloid proteins,[34] and metallic ions[5]), the intracellular partners linked

FIGURE 1. Evolution of Moore's law from 1971 to 2011. Source: https://commons.wikimedia.org/wiki/User:Wgsimon through Creative Commons Attribution-ShareAlike License (https://creativecommons.org/licenses/by-sa/3.0/).

to the GPI-binding protein PrP^C are also numerous: anti-apoptotic proteins,[35] cytoskeletal proteins,[36,37] enzymes,[34] and synaptic proteins.[38,39] For some of them clear biological relevance is still unknown, warranting further study.[27,40] Taking into account that "dubium sapientiae initium," as Descartes had it, researchers cannot state the functions or the particular role of a single protein in a specific physiological event taking as information source analysis of a transgenic mouse. In this commentary, we will summarize the current knowledge of some predicted functions of PrP^C, especially related to its putative participation in synaptic plasticity, neuroprotection, and neurodegeneration.

ZÜRICH I KNOCKOUT MICE AND THEIR PITFALLS

Revealing evidence indicates that PrP^C is not the main actor for some of the above-mentioned functions in B6129 *Prnp^{Zrchl/Zrchl}* knockout mice. In fact, a number of *Prnp*-flanking genes associated with the 129/*Sv* genotype in B6129 mixed mice have been described in Zurich I mice.[41] A ratio of 60% *to* 2% of 129/*Sv* specific markers between *Prnp^{0/0}* and *Prnp^{+/+}* mice was determined.[42] These genes were introduced during the generation of the transgenic mice and are retained in *Prnp^{0/0}* progeny of congenic B6.129 *Prnp^{Zrchl/Zrchl}* after numerous (>10 −15) crosses of B6129 *Prnp^{0/0}* with

C57BL/6 mice.[26,41] In a recent study[43] we determined, using a commercially available SNP analysis, that in backcrossed mice (5 to 6 rounds) enrichment of the C57BL/6-associated SNPs increased from ≈60% to ≈93% in the progeny. Thus, B6.129 $Prnp^{Zrchl/Zrchl}$ wild type and mutant mice may still differ at these (≈6–7%) additional 129 polymorphic loci (i.e.: *Mmu2* genomic region close to *Prnp*) accidentally present in $Prnp^{0/0}$.[44,45] This presence and parallel effects were suggested by Steele and co-workers in a seminar review published in this journal in 2007.[28] But one of the first demonstrations of the putative unwanted effects due to the presence of polymorphic 129 regions was indicated by A. Aguzzi's lab in 2010, when analyzing the effects of *Mfge8* ablation in prion infection and disease evolution in $Prnp^{o/o}$ mice.[46] In the study, the absence of *Mfge8* in a B6.129 background increases the appearance of prion disease after inoculation, in contrast to C57BL/6 $Mfge8^{0/0}$ inoculated mice.[46] In addition, Calella and coworkers described quantitatively the changes in the relative percentage of these loci after crossing.[42] Three years later, one identified gene, the signal regulatory protein α (SIRPα), was described for the first time as being responsible for a previously PrP^C-associated phagocytic function in macrophages.[45] These masking functions associated with this locus are especially relevant if we take into account that *Prnp*-overexpressing mice (Tg20) generated in mixed B6129 $Prnp^{Zrchl/Zrchl}$ background carry several copies of the polymorphic loci, since in most cases they are also crossed with previously backcrossed B6.129 $Prnp^{Zrchl/Zrchl}$ mice.[41] In this scenario, it is reasonable to assume that the data obtained using $Prnp^{o/o}$ or Tg20 in physiological studies (from genomic to electrophysiological) may render conflicting results, as observed[22,23,43,47-56] (see also[28]). However, these side effects in an undetectable manner might also occur in other mouse models with overexpressed modified forms of PrP^C using the *Prnp* null-background of the Zurich I mice.[17,57,58,59] In fact, at the transcriptional level, comparison of the mRNA expression profile reported in 2 studies revealed that B6.129 and FVB/N $Prnp^{0/0}$ mice share only

very few overexpressed genes in the adult hippocampus.[54,55] This result, as suggested,[55,60] might reflect, among other possibilities i) direct transcriptional effects of the absence of *Prnp* on different genetic backgrounds, or ii) a summation of changes linked to the deficiency of functional PrP^C together with the side effects linked to the 129-associated loci in the strains used in these studies: B6.129 $Prnp^{0/0}$ (B6129 backcrossed with C57BL/6 mice for 15 generations[54] and FVB/N mice (B6129 backcrossed with FVB/N > 20 generations).[55] In fact, some of these 129-associated loci very close to *Prnp* could be detected after ≈20 backcrossings in B6.129 mice (A. Aguzzi personal communication).

The number of these "*Prnp*-flanking genes" in the B6129 knockout mouse compared to C57BL6/J is large as demonstrated by A. Aguzzi's lab.[41] Thus, a re-evaluation of the published functional data concerning PrP^C is mandatory in order to clarify this and to delineate PrP^C functions in neural and non-neural tissues. Alternatively, researchers may study these putative functions in different biological systems in more controlled models far from the mixed mice. Indeed, in a recently published study we dissected the participation of these 129/*Sv*-associated genes in neurotransmission and kainate-induced cell death in different mouse strains and *in vitro*.[43]

INVOLVEMENT OF PRP^C IN NEUROTRANSMISSION AND NEURAL PLASTICITY: A PUZZLE BEING CLARIFIED A STEP AT A TIME

Different laboratories have described, in several $Prnp^{0/0}$ strains including congenic B6.129 $Prnp^{Zrchl/Zrchl}$, enhanced sensitivity to seizures after the administration of epileptogenic drugs such as kainic acid (kainate, KA), N-methyl-d-aspartic acid (NMDA), pilocarpine, and pentylenetetrazol (PTZ), suggesting a neuroprotective role of PrP^C against excitotoxic convulsive insults (e.g.,[22-

FIGURE 2. (A) Domain organization of PrP^C (mouse sequence). (B) Examples of some derived truncated forms used in *in vitro* and *in vivo* studies. The effects of their transfection in cells are indicated from (++) strong effect to negligible (−) effects in apoptosis. The examples of truncated PrP^C forms are summarized from.[68,94,106,107] Results obtained in several studies reinforced data obtained by D.R. Brown's Lab in 2003.[108](C) Epitope mapping of some antibodies used in cytotoxicity studies. The name and recognized PrP^C region is indicated in each case. Green antibodies indicate that their use is non-cytotoxic in contrast to red antibodies.
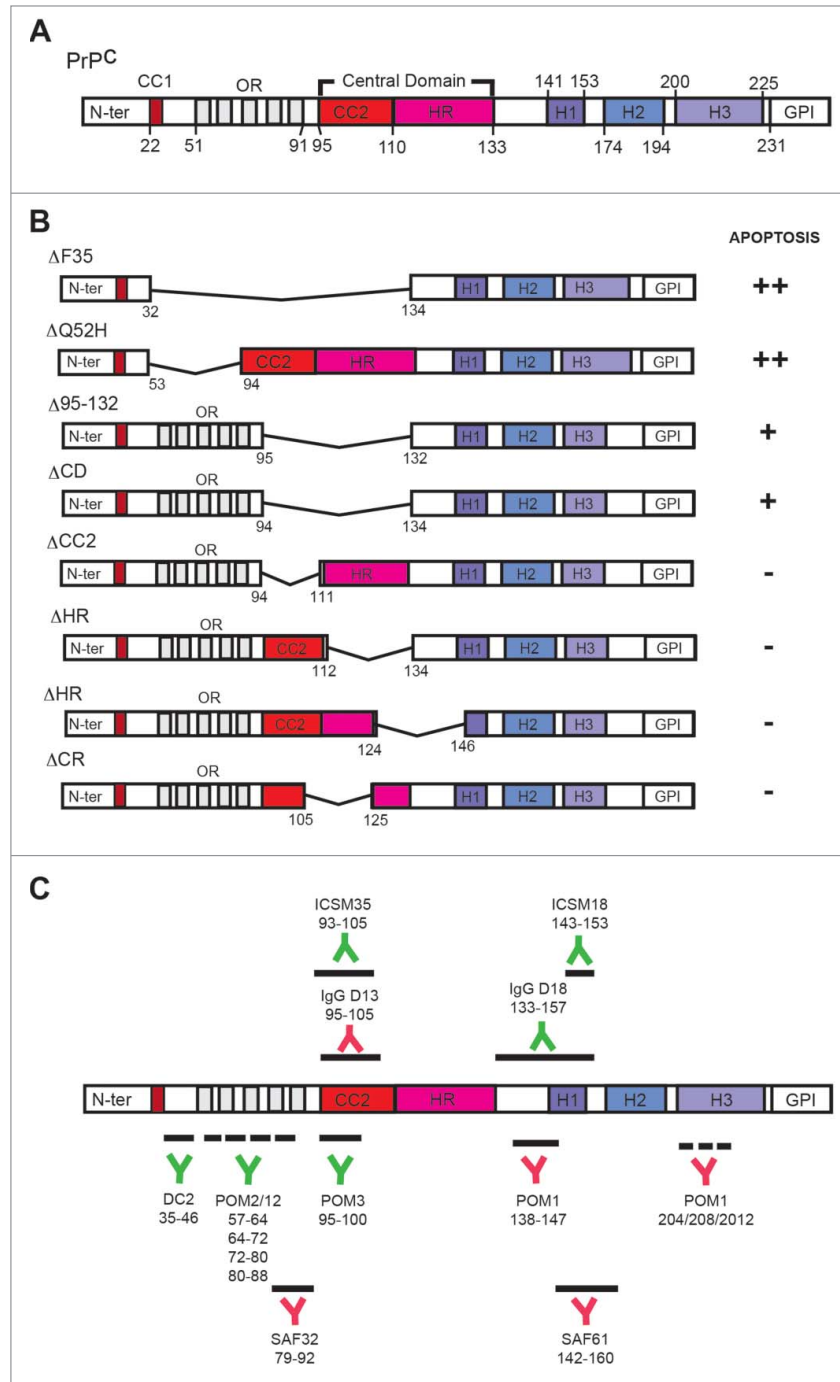
[25,40,53]). However, other studies have suggested that PrP[C] is not involved in KA-mediated excitotoxicity and that the observed differences between wild type and *Prnp*-deficient mice are associated with the genetic background of the mice used in the experiments.[51,52] In addition, an electrophysiological study indicated that PrP[C]-expressing neurons *in vitro* are more resistant against 3 different convulsive treatments (including PTZ).[50] However, this last observation, obtained using crossed B6129 + FVB mice, was not expected based on previously published data by the same group using B6129 *Prnp[0/0]* mice.[61]

Can we determine the role of PrP[C] in KA-mediated neurotoxicity in a region such us the hippocampus by using different mice strains? Our answer is affirmative, although the cytotoxic effects of KA in the mouse hippocampus are strongly strain-dependent.[62-67] Following intraperitoneal KA administration, the large majority of pyramidal neurons of the hippocampus die in the FVB/N (FVB) mouse, while the pyramidal neurons of the C57BL/6 strain remain largely healthy.[62-67] Thus it is reasonable to consider that the intrinsic background might play a role in the results reported by Ratte and coworkers.[50] Obviously these genetic differences may mask or dilute the participation of *Prnp* in KA-mediated neurotoxicity. In our study we were unable to identify specific participation of PrP[C] in KA-treated FVB/N *Prnp[0/0]* and FVB/N *Prnp^{+/+}* mice.[43] In contrast, in B6129 and B6.129 *Prnp[0/0]*, KA-mediated effects (cell death, astrogliosis, and increased presence of pro-inflammatory molecules) were identifiable, as previously reported[23,53] and corroborated using 129/Ola *Prnp[0/0]* mice[43] (whithout *Prnp*-flanking genes). In addition, these genomic influences do not explain *per se* the neuroprotective properties of PrP[C] observed in KA-treated neuroblastoma cell lines carrying different dosages of the *Prnp* gene.[43,53] In addition, regulatory participation of PrP[C] in neurotransmission and neuroprotection, and in other cellular functions, has also been demonstrated with acute modulation of *Prnp*

expression in neural cell lines (see[68]) and in other organisms (e.g., zebrafish).[69] We believe that functions of this protein in neurotransmission and neuroprotection are currently supported by i) their binding to glutamate receptor subunits (e.g.,, GluR6/7, NR2D, GluR1/2, mGluR1/5),[24,53,70,71] ii) their binding to ion channels,[49] iii) their regulation of GluR6/7- and NR2D-mediated signaling,[53] and iv) a recently published observation indicating that PrP[C] and copper cooperatively inhibit NMDA receptor through S-nitrosylation enhancing neuroprotection.[25] However, if we compare the number and level of the convulsive seizures between B6129 and B6.129 *Prnp[0/0]* mice, a clear decrease in the seizure level can be seen in parallel to a decrease in the 129-associated loci between B6129 and B6.129 mice. Using this approximation we may assume that not only PrP[C] but also unidentified 129/*Sv*-associated gene/s contribute to the KA-mediated sensitivity observed in B6129 *Prnp[0/0]* mice. Moreover, B6129 *Prnp[0/0]* mice overexpressing truncated forms of PrP[C] showed additional increased degeneration that B6129 *Prnp[0/0]* mice suggesting that susceptible neurons become highly reactive to cellular stress induced by KA, indicating that specific domains of the protein may play a role in triggering cell death in certain physiological and non-physiological conditions.

MOLECULAR DISSECTION OF THE PARTICULAR FUNCTIONS OF PRP[C] IN CELL DEATH: LESSONS FROM TRUNCATED FORMS, ANTIBODIES, AND PEPTIDES

The sequence of PrP[C] can be divided into 2 structurally well-defined regions: a long, flexible N-terminal flexible tail (approximately the first 100 residues) and a globular C-terminal domain containing 3 α-helices and 2 β-strands flanking the first α-helix (Fig. 2). The flexible tail also has distinctive features: a small charged region (CC1), an octarepeat (OR)

region, and a central domain (CD), which in turn comprises a second charge cluster (CC2) and a hydrophobic region (HR). In order to demonstrate that the N-terminal domain is mainly responsible for cooper-binding in anti-oxidative protection, Zeng and co-workers demonstrated that when the N-terminal domain of PrP^C is tethered to the plasma membrane, this modified PrP^C largely compromised cell survival due to the resulting inability to control cellular stress.[72] In fact, by using other methods (antibody treatment) it has been determined that the proximity of the flexible tail of the protein to the cellular membrane leads to cell death by activating reactive oxygen species (ROS) generation.[73,74] This has also been indicated by using transgenic mice.[59] Surprisingly, the location of cell death and its timing in the recently described FTgpi155 mouse (lacking 141 to 225 residues)[59] are rather similar to what is reported in other mice lacking domains of PrPC: i.e., ΔF35.[75] This anatomical correlation of cell death relies on the stronger activity of the *Prnp* promoter in cerebellar granule cells in these mice. However, ΔF35 mediated cell death cannot be reverted by overexpressing anti-apoptotic molecules, indicating that both caspase 3 and non-caspase 3 directed mechanisms are mediating ΔF35-associated cell death. Thus, overexpressing truncated forms of the protein in cultured cells could be also an alternative approach to determining the functions of particular domains of PrP^C in cytotoxicity. In our studies, we determined that the expression of ΔF35 and ΔCD in neuroblastoma (N2a) cells is cytotoxic and activates Caspase 3, thereby corroborating previous results[76] (see also[68]). However, only ΔCD was able to increase ROS production in transfected cells. This correlates with results using FTgpi155 mice, since in both cases the flexible tail (containing the OR) is close to plasma membrane. As indicated above, the FTgpi155 mice showed similar degeneration to ΔF35 mice; however ΔC4 mice (lacking) do not display cerebellar degeneration but are more susceptible to ischemic insults with increased ROS generation.[77] Concerning the effects of antibodies we need to consider several scenarios. First, antibodies (e.g., SAF61, recognizing residues 142 to 160 of PrP^C) are

able to aggregate PrP^C in the plasma membrane, activating fyn at lipid rafts and triggering cell death.[9] In this process the activity of ERK1/2 and NADPH oxidase plays a crucial role.[78,79] Indeed, the use of similar antibodies against PrP^C *in vivo* also leads to cell death.[80] The antibodies used in the *in vivo* experiments were IgG P and IgG D13 (recognizing residues 95 to 105 region of the PrP^C). Second, antibodies directed to the globular domain are able to induce cell death *in vitro* and *in vivo* independently of PrP^C aggregation.[73,74] In contrast, the injection of the ICSM35 antibody (recognizing PrP^C epitopes 93 to 105) and ICSM18 (recognizing residues 143 to 153 of PrP^C) failed to induce cell death in the hippocampus of C57BL10 mice.[81] In fact, it has been observed that the use of antibodies (POM1) directed to the globular domains triggers similar neurotoxic responses to those of pathogenic prion by approaching the flexible N-terminal domain to the plasma membrane.[74]

Some years ago, the use of synthetic peptides was revealed as a new alternative for analyzing prion neurotoxicity.[82] In fact one of the most widely used peptides of PrP$_{(106-126)}$ with aggregating properties needed the terminal half of the HR region but not the OR in order to be neurotoxic.[83] This result was in line with a previously published manuscript indicating that this peptide is able to induce ROS production in cultured neurons,[84] which could also be increased by overexpressing PrP^C.[85] In fact, mice lacking the aa 105–125 of PrP^C showed early death during the postnatal period.[86] Thus, although the existence of a survival signal interacting at this level or PrP^C to trigger neuroprotective signals could not be ruled out, one should be tempted to strongly consider Ockham's razor theory in this scenario: "*Among competing hypotheses, the one with the fewest assumptions should be selected.*" It may be that these antibodies acting on the globular domain, the infective prion, or the mimicking peptides are blocking the intrinsic activity of the N-terminal domain, as happens when the domain is approached or tethered to the plasma membrane. Thus, the homeostatic cellular function of PrP^C could be lost, triggering a cell-death mechanism (Fig. 3). However, we should

FIGURE 3. Scheme illustrating the effect of the expression of particular truncated forms of PrP^C (B-E), treatment with GD-directed antibodies (F), peptides recognizing the CD region (G), aggregating antibodies recognizing GD and OR regions (H), and pathogenic prion protein (I). Absence of the OR in B and C leads to increased apoptosis. In contrast, PrP^C lacking the CD but more relevantly lacking both the GD and the CD induced increased neurotoxicity. In contrast, aggregating antibodies (H), GD-directed antibodies (F), peptides (G), and the pathogenic prion (I) lead to profound changes in the 3D organization of PrP^C in the membrane, which triggers the approach of the N-terminal region to the plasma membrane (red curved arrow) leading to increased ROS production and cell death as observed in PrP^C constructs with artificial tethering of the N-terminal to the membrane. In these conditions, PrP^C recycling is very low and their homeostatic function is lost.
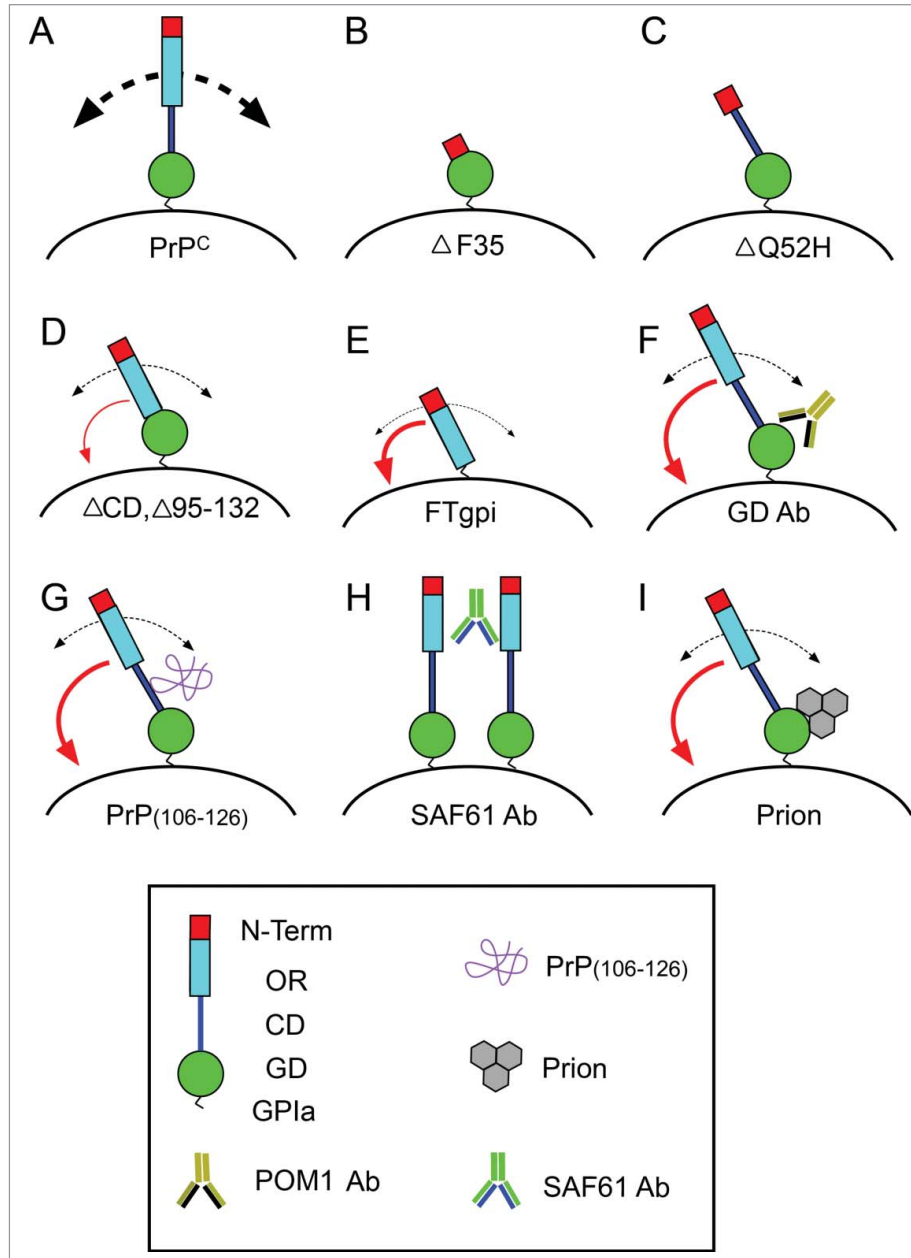
forget that other perspective was offered by Walter Chatton: "*If 3 things are not enough to verify an affirmative proposition about things, a fourth must be added, and so on.*" The coming years and experiments will reveal whether we should apply Ockham's razor, or not, concerning PrP^C-mediated cell death.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

Examples of Pubmed citations on PrP^C functions

Paragraphs illustrating 3 examples of manuscripts describing some functions of the PrP^C.

These manuscripts were selected as examples with the Pubmed search of the subject "cellular prion protein" and the particular process as keyword. As examples, the keywords cell survival, neurotoxicity and Alzheimer's disease were used. Notice that in each description several studies reported roles for PrP^C at different levels, and in some cases these are controversial.

Cell survival and cellular prion protein

PrP^C increase cell proliferation and survival[1]

PrP^C absence increases proliferation of hippocampal precursors[26]

PrPC mediate cell cycle on neuroblastoma cells[2]

Cell survival by acting on PIK3 kinase[87]

Cell survival promoting glucose uptake in cancer cells[88]

Cell survival after genotoxic stress[89]

Yeast prion promote drug resistance and cell survival[90]

Neurotoxicity induced by the cellular prion protein or its domains *in vitro* and *in vivo*

A synthethic peptide of PrP^C is neurotoxic[79,82]

Description of the neurotoxicity of the putative transmembrane domain of PrP^C.[91]

Description of the neurotoxic potential of the cytoplasmic domain of PrP^C[92]

Peptides mimicking the central domain of PrP^C triggers cell-death pathways[93]

PrP^C aggregating antibodies induced cell death[9]

Membrane tethered PrP^C triggers cell death in mice[59]

The flexible tail of PrP^C mediates cell-death induced by PrP antibodies[73]

PrP^C-directed antibodies do not trigger apoptosis[81]

Distinct domains of PrP^C triggers different intracellular cell-death signals[94]

Cellular prion protein and Alzheimer's disease (Aβ).

PrP^C–derived peptides bind to amyloid precursor protein (APP)[95]

PrP^C regulates BACE1 activity[34] and APP processing[84]

PrP^C mediates impairment of synaptic plasticity mediated by Aβ[96,97]

PrP^C mediates the toxicity of Aβ oligomers[98,99]

PrP^C is not involved in Aβ-mediated toxicity and synaptic plasticity[42]

PrP^C expression is needed for memory impairment in mouse models of Alzheimer disease[100]

PrP^C immunotargeting *in vivo* prevents Aβ-mediated LTP inhibition[101]

The N-terminal domain of PrP^C binds to Aβ oligomers[102]

PrP^C modulate Aβ production and deposition in mouse models[103]

Metabotropic glutamate receptor 5 is a coreceptor of Aβ oligomers and PrP^C[104]

Blocking Aβ binding to PrP^C as terapeuthic strategy for Alzheimer's disease.[105]