# Darwinian Evolution of Prions in Cell Culture

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Prions are infectious proteins consisting mainly of $PrP^{Sc}$, a β sheet–rich conformer of the normal host protein $PrP^{C}$, and occur in different strains. Strain identity is thought to be encoded by $PrP^{Sc}$ conformation. We found that biologically cloned prion populations gradually became heterogeneous by accumulating "mutants," and selective pressures resulted in the emergence of different mutants as major constituents of the evolving population. Thus, when transferred from brain to cultured cells, "cell-adapted" prions outcompeted their "brain-adapted" counterparts, and the opposite occurred when prions were returned from cells to brain. Similarly, the inhibitor swainsonine selected for a resistant substrain, whereas, in its absence, the susceptible substrain outgrew its resistant counterpart. Prions, albeit devoid of a nucleic acid genome, are thus subject to mutation and selective amplification.

Prions are the infectious agents responsible for a variety of neurodegenerative disorders, including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and new variant Creutzfeldt-Jacob disease (CJD) and kuru in humans. The principal, if not only, component of the prion is $PrP^{Sc}$, a β sheet–rich conformer of prion protein, (PrP). $PrP^{Sc}$ propagates by eliciting conversion of $PrP^{C}$, the physiological form of PrP, into a likeness of itself. The seeding hypothesis posits that $PrP^{C}$ is in equilibrium with $PrP^{Sc}$ or a $PrP^{Sc}$ precursor, with the equilibrium largely in favor of $PrP^{C}$, and that $PrP^{Sc}$ is only stabilized when it forms an aggregate, or seed, containing a critical number of monomers. Once a seed is present, monomer addition ensues rapidly (*1*).

Prions occur in the form of distinct strains, originally characterized by the incubation time and the neuropathology they elicit in a particular host (*2*). Many different strains can be propagated indefinitely in hosts homozygous for the PrP gene (*Prnp*); the protein-only hypothesis assumes that each strain is associated with a different conformer of $PrP^{Sc}$ (*3*–*5*), which implies that there are as many stable conformations of PrP as there are stable prion strains that can be propagated in a particular mouse strain, perhaps 15 or more (*6*). The concept of "conformation templating" at the protein level was first supported by cell-free conversion experiments (*7*) and extended by the development of protein misfolding cyclic amplification (PMCA), which mimics $PrP^{Sc}$ autocatalytic replication in vitro (*8*).

A prion strain, transferred from one species to another and subsequently returned to the original host, may in some instances have changed or "mutated" (*9*, *10*). Novel strains may arise not only by mutation of naturally occurring strains, but also de novo, in transgenic mice (*11*) or in cell-free systems, mediated by PMCA (*12*).

Strains are classically differentiated by mouse bioassays that require months or years to complete. The cell panel assay (CPA) (*13*) allows assessment of the characteristic cell tropism of strains by the standard scrapie cell assay (SSCA) (*14*) on a panel of four cell lines, the neuroblastoma-derived PK1 and R33, the neuronal CAD, and the fibroblastic LD9 lines. The CPA readily distinguishes between RML, 22L, ME7, and 301C prions within about 2 weeks.

We have found that 2 μg/ml (11.55 μM) swainsonine (swa), an inhibitor of Golgi α-mannosidase II that impairs formation of complex N-linked glycans, inhibits by 99% or more chronic infection of PK1 cells by RML and 79A but not by 22L prions. The median effective dose for inhibition of infection is 3 ng/ml. The misglycosylation of host proteins caused by swa has no effect on cell growth but reduces the accumulation rate of "swa-sensitive," but not of "swa-resistant," prions below the replication rate of the host cells, which causes the prions to be diluted out with progressive cell doublings.

It has been reported that strain specificity is retained when prions are transferred from brain to cultured cells and back to brain (*15*, *16*); however, the properties of the prions while in cell culture could not be determined by classical procedures. We therefore examined prion characteristics using the CPA. We generated a chronically infected cell population, $PK1[22L]_{wp}$, by exposing PK1 cells to 22L-infected mouse brain homogenate (brain[22L]) and propagating them for about 34 doublings. The CPA showed that cell-derived and brain-derived prions differed: The cell-derived prions were unable to infect R33 cells ("R33 incompetent") or to infect PK1 cells in the presence of swa ("swa sensitive"), in contrast to brain[22L]-derived prions, which were able to do both. (Fig. 1, A and C). We considered that cell- or brain-derived components might influence the infectious properties of the prions on the cell panel. If this were so, the full change in properties would be observed immediately after the first round of prion replication in the cells. We therefore infected PK1 cells with 22L-infected brain homogenate and collected conditioned medium 9 days after infection (P0) and after successive 1:10 splits. The CPA (Fig. 1B) showed that, at P0, the secreted prions resembled brain prions, in that they were R33 competent and swa resistant, and that with successive splits they became less infectious to R33 and more susceptible to inhibition by swa. By the 12th 1:10 split, i.e., after about 40 doublings, the properties of the population were indistinguishable from those of the $PK1[22L]_{wp.}$ Thus, when transferred from brain to PK1 cells, the prion population underwent a gradual, not a sudden, change in properties. This suggested that the brain-derived population might be heterogeneous and that the R33-competent, swa-resistant prions that predominated in brain were replaced by R33-incompetent, swa-sensitive prions with a growth advantage in PK1 cells.

Having established that the prions in $PK1[22L]_{wp}$ cells were swa sensitive, we attempted to cure the cells of infection by propagating them in the presence of swa for ten 1:20 splits (Fig. 2, A and B). In the absence of swa, the percentage of $PrP^{Sc}$-positive cells remained essentially unchanged, between 30 and 40%. In the presence of swa, the percentage initially dropped, from 35% to about 7%, but then, unexpectedly, increased to reach a value of about 25% by the 8th to 10th split, which suggested the development of resistance. To investigate this in more detail, we analyzed the swa susceptibility of prions secreted by $PK1[22L]_{wp}$ cells. Prions from cells propagated in the absence of swa were swa sensitive at all splits tested (Fig. 2C). In contrast, prions from cells propagated in the presence of swa for two or more splits were completely swa resistant. In addition, $PK1[22L]_{wp}$ cells grown for five passages in the presence of swa and then for five splits in its absence secreted prions that were again fully swa sensitive. Thus, in the presence of the drug, preexisting or newly generated swa-resistant prion variants selectively grew to dominate the population. Moreover, after withdrawal of the inhibitor, residual or newly generated swa-sensitive prions replaced their swa-resistant counterparts almost completely after about 22 doublings, presumably because, in the absence of swa, the drug-sensitive prions replicated more rapidly. We confirmed all these results by assaying lysates of the same cell cultures described above (*17*). Moreover, the entire experiment was repeated by a different operator with the same results.

Swa prevents normal complex glycosylation of N-linked glycans (*18*), and the resulting high-mannose glycans are cleavable by endoglycosidase H (Endo H), in contrast to native complex glycans, which are completely resistant. Treatment of proteinase K (PK)–digested samples from control $PK1[22L]_{wp}$ cells (Fig. 2D) with Endo H

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did not result in PrP bands with increased mobility. However, after the first 1:20 split (about 4.3 doublings) in swa-containing medium (Fig. 2D), there was a significant mobility shift of all bands due to the loss of complex glycosylation, and Endo H treatment caused a dramatic increase in mobility. Yet, as described above (Fig. 2C and table S1), the prions from this sample were still swa sensitive, which showed that the lack of complex glycosylation was not the cause of swa resistance. When swa treatment was discontinued, the mobility pattern reverted to that of untreated controls after two 1:20 splits [SC7 in (Fig. 2D)], and the glycans were completely resistant to Endo H. However, the prions continued to be swa resistant until after the fifth split (SC10). Thus, lack of complex glycosylation is not responsible for swa resistance, and normally glycosylated $PrP^{Sc}$ can be associated with both swa-sensitive and swa-resistant prion variants.

Some prion strains differ in the site at which the cognate $PrP^{Sc}$ is cleaved by PK (*3*, *4*, *19*) or thermolysin (*20*) or in the rate at which it is degraded (*21*). We treated lysates of swa-sensitive and swa-resistant PK1[22L]$_{wp}$ cells with PK or thermolysin (fig. S3) and detected no significant differences in the electrophoretic mobility of the cleavage products. There was no significant difference in the susceptibility of $PrP^{Sc}$ from brain[22L], swa-sensitive, or swa-resistant PK1[22L]$_{wp}$ cells to PK (fig. S4). $PrP^{Sc}$ from different strains may exhibit different stabilities, as determined by susceptibility to PK digestion after exposure to increasing concentrations of guanidinium chloride (Gnd.HCl) (*11*, *22*). We subjected lysates of swa-sensitive and swa-resistant PK1[22L]$_{wp}$ cells, as well as homogenates of brain[22L], to the conformational stability assay, but found no significant differences in the Gnd.HCl$_{1/2}$ values, which were 1.1 to 1.3 M in all cases (fig. S5). These negative findings suggest that structural differences between the postulated substrains may be discrete.

Cell lysates were injected intracerebrally into C57BL/6 mice, and brains were recovered when the mice became terminally ill at 147 days after inoculation (table S2). The CPA of authentic brain[22L] and the brain-passaged swa-sensitive and swa-resistant PK1[22L]$_{wp}$ prions gave indistinguishable patterns (Fig. 1C and fig. S6), showing that the PK1-derived 22L prions regained their original cell tropism after propagation in brain. Brain sections revealed the vacuolization of the granular layer of the cerebellum and the loss of Purkinje cells typical for 22L for both cell-derived (swa-sensitive and swa-resistant) and brain-derived samples (fig. S7 and table S3).

The finding that exposure of 22L-infected PK1 cells to swa leads to the emergence of swa-resistant prions means that such variants either exist in the population at a low level before exposure to swa, or they are generated during exposure to the drug. To address this question, we exposed PK1 cells to PK1[22L]$_{wp}$ prions in either the presence or absence of swa for 2 days and distributed the cells into 96-well plates at 8 cells per well for the cells infected in the absence of swa and 1000 cells per well for cells infected in the presence of swa (fig. S2). Uninfected cells were added to bring the total number of cells in each well to 1000. The cells were grown to confluence and split 1:10 five times, in the continued absence or presence of swa. Under these conditions, any well containing one or more infected cells yields a positive signal in the PK-digested enzyme-linked immunosorbent assay (PK-ELISA) (*17*) because of the continuous spread of infection (*14*). The average number of infected cells delivered to each well was calculated by the Poisson equation. Of the wells from the swa-exposed population, 0.029% were $PrP^{Sc}$-positive, and from the non–swa-exposed population, 5.8% were $PrP^{Sc}$-positive (fig. S2). Because swa inhibits replication of PK1[22L]$_{wp}$ prions by more than 99%, these results indicate that 0.5% of the prions secreted by PK1[22L]$_{wp}$ cells were swa resistant before exposure to the drug.

The 22L isolate obtained from the TSE Resource Center (Compton, UK) had been biologically cloned twice in succession (*23*), yet here we found it to be heterogeneous with regard to its swa sensitivity after transfer to cultured cells, which suggested that variants had arisen during the two rounds of propagation in mice and the transfer to

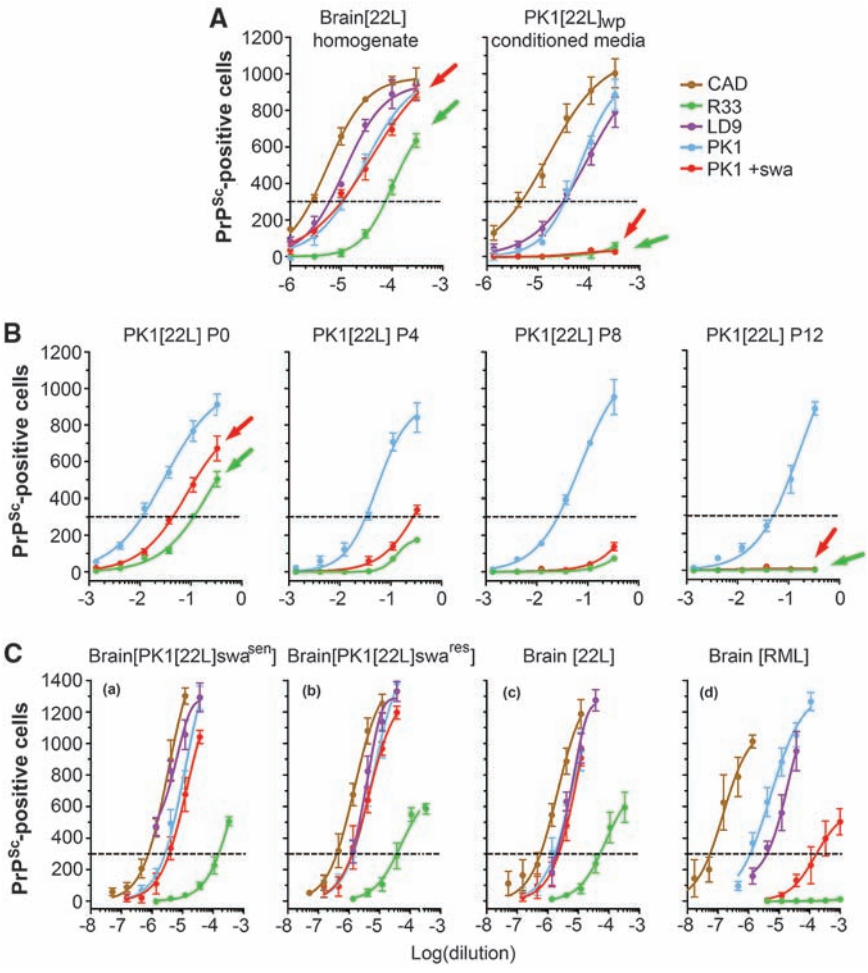


**Fig. 1.** Prion characterization by the CPA. Cells were exposed to the serially diluted samples indicated. The proportion of $PrP^{Sc}$-positive cells was plotted against $\log_{10}$ dilution. (**A**) Brain[22L]-derived prions are swa resistant and R33 competent, whereas those from 22L-infected PK1 cells (about 27 doublings after infection) are swa sensitive and R33 incompetent. (**B**) Transfer of prions from brain to PK1. PK1 cells were exposed to $10^{-4}$ brain[22L] for 1 day and propagated 9 days (P0); further propagation for twelve 1:10 splits yielded P1 to P12. Conditioned media were analyzed on PK1 cells with (red) or without (blue) 2 μg/ml swa and on R33 cells (green). Infection in the presence of 10 μg/ml pentosan polysulfate, which abolishes prion replication, yielded no infectivity, documenting the absence of inoculum. (**C**) Brain homogenates from terminally ill C57BL/6 mice inoculated with lysates of PK1[22L]$_{wp}$ cells propagated in the absence (a) or presence (b) of swa or with (c) 1% 22L-infected brain homogenate gave the same CPA responses. (d) The very different CPA response of brain RML is shown for comparison.

cells. To ascertain whether cloned prion populations could become heterogeneous, we recloned 22L prions by end-point dilution in cell culture (*16*) and analyzed the populations after various extents of propagation (Fig. 3). Eight clonal populations were assayed after about 31 doublings following infection and found to secrete swa-sensitive prions (fig. S8). Aliquots of each population were propagated for 22 doublings in the presence or absence of swa; two of the eight clones, *8C4* and *3C6*, secreted swa-resistant prions after being grown in the presence, but not in the absence, of the drug (Fig. 3A). The other six cell clones secreted swa-sensitive prions (Fig. 3A) and lost infectivity after being propagated for five 1:20 splits in the presence of swa (Fig. 3A). Three of the six clones (*8A8*, *8B4*, and *8H6*) were propagated for an additional 22 doublings in the absence of the drug [(b) to (c1) in Fig. 3A], altogether about 53 doublings, and were then exposed for 22 doublings to swa, whereupon one more clone (*8A8*) produced swa-resistant prions (Fig. 3A). Thus, swa-resistant variants arose during propagation of cloned, swa-sensitive prion populations for as few as 31 doublings in the absence of the drug. From these data, we calculated (*17*) a very approximate "mutation rate" of $10^{-6}$ per doubling. This number is an underestimate, mainly because we considered neither the selective disadvantage of swa-resistant prions in the absence of swa nor the "reversion rate."

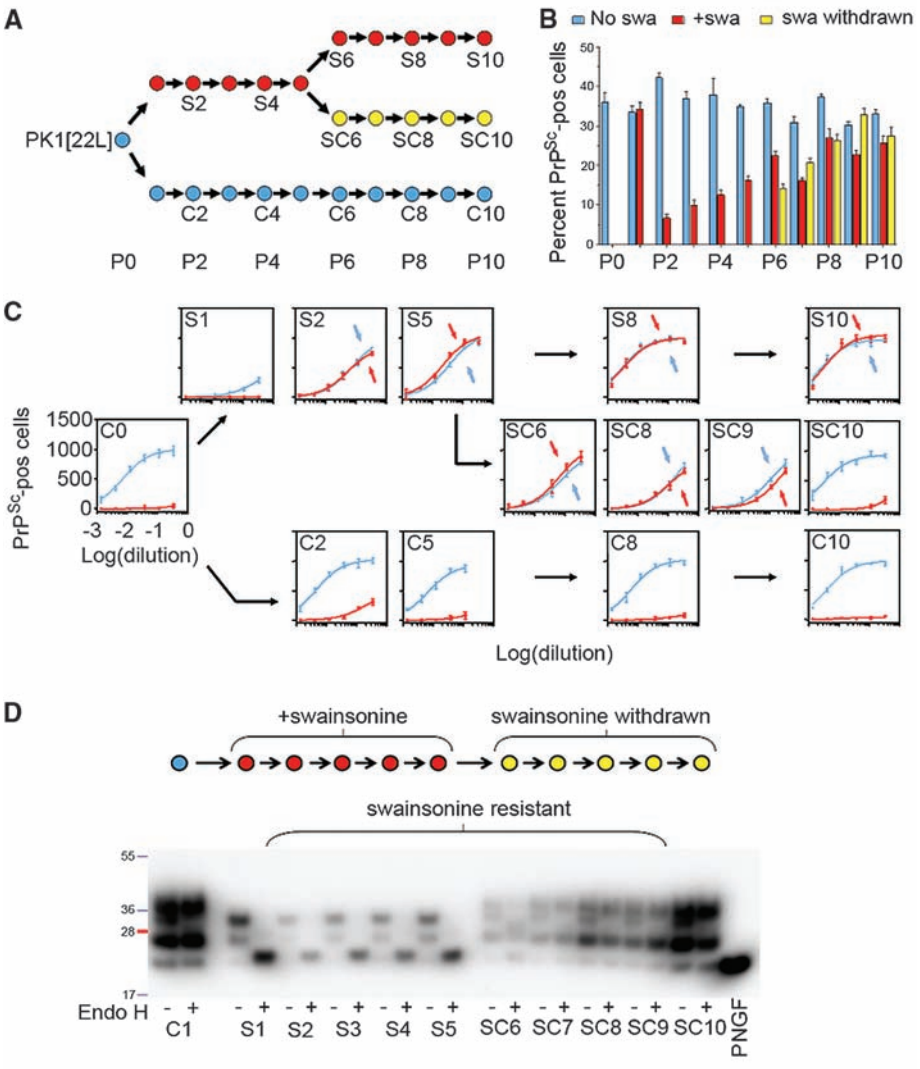


**Fig. 2.** Propagation of 22L-infected PK1 cells in swa results in swa-resistant prions. (**A**) Scheme. $PK1[22L]_{wp}$ cells were either grown in swa (2 μg/ml) for up to 10 splits (S1 to S10), without swa for up to 10 splits "C0-C10", or with swa for 5 splits followed by without swa for 5 splits (SC6 to SC10). (**B**) The percentage of $PrP^{Sc}$-positive cells propagated with swa (red) or without swa (blue, yellow), as assessed by PK-ELISA. (**C**) Swa susceptibility of secreted prions propagated in the presence or absence of swa. Conditioned medium, concentrated 100×, was assayed on PK1 cells in the presence (red) or absence (blue) of swa. (**D**) Swa-resistant prions are associated with both $PrP^{Sc}$ carrying high-mannose glycans (S2 to S5) and normally glycosylated $PrP^{Sc}$ (SC7 to SC8). $PK1[22L]_{wp}$ cell lysates digested with PK and Endo H were subjected to immunoblotting with antibody against PrP. After the first split in swa, PrP bands shift to higher mobility, which reflects the inhibition of complex glycosylation, and Endo H digestion results in a large mobility increase due to removal of high-mannose glycans. After two splits without swa (SC7), normal glycosylation is restored but swa resistance is retained.

Perhaps swa resistance and R33 competence are only two of many variations that can arise in a 22L population; if so, the overall mutation rate could be even greater and the prion population more diverse, comprising a multiplicity of "substrains" or "types" (*24*). This would be reminiscent of the "quasispecies" concept (*25*, *26*). Although heterogeneity in the case of RNA viruses is due to point mutations resulting from error-prone replication, heterogeneity in the case of the prions is likely to be due to differences in the structure (other than the amino acid sequence) of the $PrP^{Sc}$ molecules. These differences could reflect variations in the conformation of the $PrP^{Sc}$ resulting during conversion; the conformational changes may be subtle, but sufficient to facilitate propagation in a particular environment. Alternatively, or in addition, variation could be due to cell-derived determinants (for example, association of PrP with a small cellular RNA) or to the nature of its glycosylation.

Transfer of a prion strain from one animal species to another usually entails a low attack rate and long incubation times, which in subsequent passages are dramatically reduced (*27*). If the only barrier to prion transfer between species were the initial round of heterologous conversion, then once it occurred, propagation would be rapid. However, this is almost never so, and two or three sequential transmissions are required to obtain stable, shortened incubation times, which suggests that additional, likely conformational, changes are required to optimize prions for propagation in the new host (*9*, *10*, *28*). It has also been argued that even within a single host, different "strain types" may develop within different tissues (*29*–*31*), and the "cloud" model to explain these observations (*24*) is well supported by our findings.

In what ways do the concepts of strains and substrains differ? The energy landscape diagram of fig. S10 depicts the view that substrains are distinct collectives of prions that can interconvert reproducibly and relatively readily, that is, within the generation time of the host or a few dozen rounds of replication, because they are separated by relatively low activation energy barriers; strains, each comprising a set of readily interconvertible substrains, are separated by higher energy barriers, causing transitions to be rare events.

In summary, prions show the hallmarks of Darwinian evolution: They are subject to mutation, as evidenced by heritable changes of their

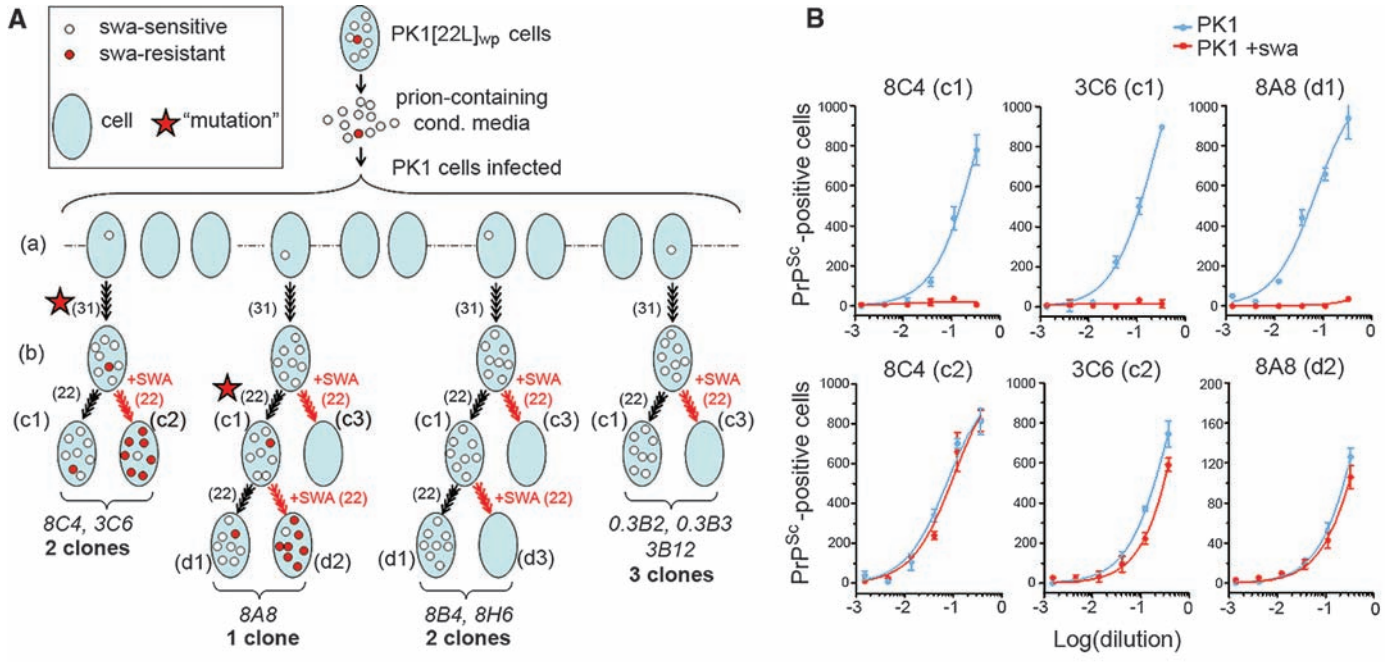


**Fig. 3.** Development of heterogeneity in cloned prion populations. Prions were cloned by end-point dilution in cell culture, and eight clones were tested for their ability to yield swa-resistant populations after propagation in swa. Values in parentheses indicate number of doublings. (**A**) PK1 cells were exposed to PK1[22L]$_{wp}$ conditioned medium for 2 days, which led to infection of about 4% of the cells. Cells were distributed at 0.3, 1, 3, or 8 cells per well (a), along with about 1000 uninfected cells, grown to confluence (7 doublings) and split 1:10 five times (17 doublings) in 96-well plates. Of 602 wells, 23 scored positive by PK-ELISA. Eight clones were expanded for seven doublings, and all secreted swa-sensitive prions [(b) altogether 31 doublings]. After propagation in swa for five 1:20 splits, clones *8C4* and *3C6* yielded swa-resistant prions (c2), whereas the others were cured (c3). Propagation without swa yielded swa-sensitive prions (c1). Three of six clones that failed to yield swa-resistant populations after exposure to swa (8A8, 8B4, and 8H6) were further passaged for 22 doublings without swa (c1), followed by 22 doublings with swa, whereupon one (*8A8*) yielded swa-resistant prions (d2), and two (8B4 and 8H6) were cured (d3). Details in (*17*). (**B**) Swa resistance was determined by assaying 100× concentrated conditioned medium on PK1 cells with or without swa.

phenotypic properties, and to selective amplification, as documented by the emergence of distinct populations in different environments. A practical consequence of our findings is the realization that therapeutic approaches aimed at stabilizing PrP or reducing PrP expression are less likely to be thwarted by emergence of drug resistance than those based on targeting $PrP^{Sc}$.

*Note added in proof*: Exposure of mice or differentiated neuroblastoma cells infected with RML prions to quinacrine leads to drug-resistant prions (*32*).

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33. We thank C. A. Demczyk for assistance with cell culture, E. Smith for assistance with the SSCA, I. Suponitsky-Kroyter for the proteolytic digestion analyses, and A. Sherman for histochemistry and animal work. We thank T. Bartfai, G. Joyce, and C. Lasmezas for critical reading of the manuscript and valuable suggestions. The project was supported by an R01 grant (#NSO59543) from the NIH and by a generous donation from the Alafi Family Foundation to C.W.


### Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S10
Tables S1 to S4
References


12 October 2009; accepted 17 December 2009
Published online 31 December 2009;
10.1126/science.1183218
Include this information when citing this paper.

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*Science* **327** (5967), 869-872.
DOI: 10.1126/science.1183218originally published online December 31, 2009

**DNA-less Evolution**

Prions are proteinaceous infectious elements involved in a variety of neurodegenerative diseases, including scrapie in sheep and so-called mad cow disease in cattle. Now **Li *et al.*** (p. 869, published online 31 December) show that, when propagated in tissue culture cells, cloned prion populations become diverse by mutational events and can undergo selective amplification. Thus, even though devoid of a coding genome, prions, when propagated under a particular selection regime, can be subject to rapid evolution.