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Mutability of prions

Jiali Li, Sukhvir P. Mahal, Cheryl A. Demczyk & Charles Weissmann[+]
Department of Infectology, Scripps Florida, Jupiter, Florida, USA

Murine prions transferred from brain to cultured cells gradually adapt to the new environment. Brain-derived 22L prions can infect neuroblastoma-derived PK1 cells in the presence of swainsonine (swa); that is, they are 'swa resistant'. PK1 cell-adapted 22L prions are swa sensitive; however, propagation in swa results in selection of swa-resistant substrains. Cloned, PK1 cell-adapted 22L prions were initially unable to develop swa resistance ('swa incompetent'); however, after serial propagation for 30–90 doublings, four of nine clones became swa competent, showing that swa-resistant 'mutants' arose during replication. Mutations in the case of prions are attributed to heritable changes in PrP^{Sc} conformation. One clone remained swa incompetent even after 10^{35}-fold expansion; surprisingly, after propagation in brain, it yielded swa-resistant prions, indistinguishable from the original 22L population. Thus, cell-adapted 22L prions assumed either mutable or virtually immutable conformations; however, when passaged through the brain all became mutable. Mutability is thus a substrain-specific attribute.

Keywords: mutation; selection; strain; substrain; swainsonine
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INTRODUCTION

The agent causing transmissible spongiform encephalopathies, the prion, consists mainly, if not entirely, of PrP^{Sc}, an aggregated conformer of the ubiquitous host protein PrP^{C} (Colby & Prusiner, 2011). PrP^{Sc} is thought to replicate by 'seeded polymerization', a process by which PrP^{C} accretes to PrP^{Sc} and in doing so assumes its conformation (Gajdusek, 1988; Jarrett & Lansbury, 1993).

Prions occur in the form of distinct strains, which were originally distinguished by incubation time on various mouse lines and the lesion profile they engendered in brain, and subsequently by characterization of the physicochemical properties of the cognate PrP^{Sc}, such as mobility after proteinase K (PK) treatment (Bessen & Marsh, 1992), stability to denaturation (Peretz *et al*, 2001) and reactivity to antibodies (Safar *et al*, 1998). We have developed the cell panel assay (CPA), which characterizes murine strains by their relative capacity to infect a panel of cell lines that includes the murine neuroblastoma N2a-derived lines PK1 and R33 (Mahal *et al*, 2007). PK1 cells are susceptible to RML and 22L prions, whereas R33 cells are susceptible to 22L but not to RML prions.

Strains such as 79A, ME7 or 22L are stable and do not interconvert. In contrast, 'substrains' of a particular strain interconvert readily as they adapt to a changed environment (Li *et al*, 2010). Thus, when brain-derived 22L prions ('brain[22L] prions') were propagated extensively in neuroblastoma-derived PK1 cells, the CPA characteristics of the resulting prions ('PK1[22L] prions') changed, but were restored when the prions were again propagated in the brain (Li *et al*, 2010). PK1[22L] prions, in contrast to brain[22L] prions, are 'swainsonine (swa) sensitive', that is, their capacity to infect PK1 cells is inhibited by swa, and 'R33 incompetent', that is, they are unable to infect R33 cells. Swa is an inhibitor of α-mannosidase II, which causes misglycosylation of *N*-linked glycans and inhibits infection of PK1 cells by some prion strains (Browning *et al*, 2011). Unexpectedly, when 22L-infected PK1 cells were propagated in the presence of swa for nine doublings, the prion population became swa resistant (Li *et al*, 2010). We found that already before swa exposure the PK1[22L]-derived prion population contained about 0.5% swa-resistant variants, showing that pre-existing variants were selected when the prions were propagated in the presence of the inhibitor (Li *et al*, 2010). Because the 22L prion stock had been cloned by end point dilution in mice, the swa-resistant variants must have originated between the time of cloning and our experiments. To determine the frequency of the appearance of swa-resistant prion variants, we generated eight prion clones by end point dilution in cell culture, propagated them for various numbers of doublings and assessed the populations for 'swa competence', that is, their ability to give rise to swa-resistant populations when challenged with swa.

All cloned populations were initially swa sensitive and swa incompetent, that is, they did not develop swa resistance when exposed to the drug, indicating that the population did not contain swa-resistant mutants early on. Within 31 doublings two and within 53 doublings a third cloned population was able to develop swa resistance when challenged, that is, they became swa competent, showing that in the course of propagation swa-resistant 'mutants' had arisen in three of the eight clones (Li *et al*, 2010). The other five cloned populations remained swa incompetent, raising the question as to whether these populations were inherently incapable of giving rise to swa-resistant variants, or whether they had not been propagated long enough to allow such mutational events to occur.

Department of Infectology, Scripps Florida, 130 Scripps Way, Jupiter, Florida 33458, USA
[+]Corresponding author. Tel: +1 561 228 3459; Fax: +1 561 228 3099;
E-mail: charlesw@scripps.edu

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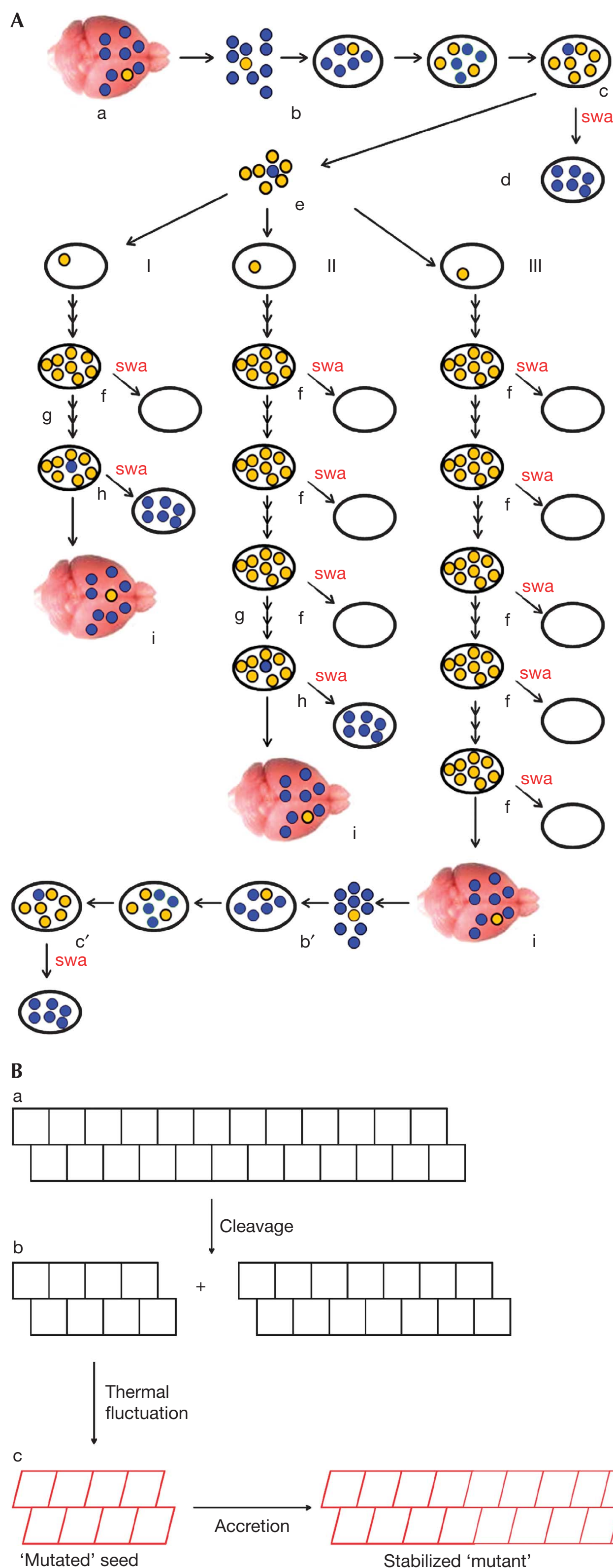

Fig 1 | Mutations in cloned prion populations. (**A**) Scheme of the experimental strategy. 'a' represents a mouse brain containing swainsonine (swa)-resistant (blue filled circles) and swa-sensitive (orange filled circles) cells. The ovals represent PK1 cells. Pathway I represents the behaviour of clones #1 and #2, pathway II that of #3 and #4 and pathway III that of #9. The events are explained in the text. Part of the scheme is based on data from Li *et al* (2010). (**B**) Conjectural mechanism of prion mutation. (a) A large aggregate of PrP^{Sc} is cleaved to smaller fragments (Orgel, 1996). (b) Small aggregates are susceptible to thermally induced conformational fluctuations. (c) A conformationally altered small aggregate is stabilized by accretion of PrP (prion protein).

This paper reports that after propagation of these five swa-incompetent populations for another 33 doublings one more clone became swa competent, whereas two did not and two remained doubtful. A further swa-incompetent clone, cdB8 (#9), isolated from 22L-infected brain homogenate, remained swa incompetent after more than 116 doublings. We found that some PK1-adapted 22L prion clones appeared to be immutable with regard to their swa competence as long as they were maintained in PK1 cells; however, after passaging through the brain they regained swa resistance and mutability, and resembled the original 22L (see scheme in Fig 1A).

RESULTS

We generated eight clones of 22L prions by end point dilution of 22L-infected PK1 cell supernatant (#1–8, Table 1 in Li *et al* (2010)) in PK1 cells, and two clones by end point dilution of 22L-infected brain homogenate (Methods section) in PK1 cells, of which one, #9 (cdB8), was further examined. The cloned populations were propagated in the absence of swa for the indicated number of doublings (31–116) and their 'swa competence', that is, their ability to give rise to swa-resistant populations under the conditions of our assay, was tested by exposing the populations to 2 μg/ml swa for 22 doublings and then subjecting them to the standard scrapie cell assay (SSCA) on PK1 cells in the presence or absence of swa. The SSCA is performed in a 96-well format by exposing susceptible cells to prions, propagating them for three 1:10 splits and then determining the proportion of PrP^{Sc}-positive cells by subjecting the sample to proteinase K (PK)-digestion followed by an enzyme-linked immunosorbent assay (PK-ELISA; Klohn *et al*, 2003).

All clones were swa sensitive when tested after 31 doublings. As shown in Table 1, after 31 doublings, clones #1 and #2, but none of the others, were swa competent; clone #3 became swa competent after 53 doublings (Li *et al*, 2010) and clone #4 after 86 doublings. The status of clones #5 and #6 after 86 doublings was questionable because infectivity had dropped to insignificant levels for clone #5 and swa resistance was borderline for clone #6 (Fig 2). Clones #7 and #8 were still swa incompetent after 86 doublings, whereas #9 was incompetent even after 116 doublings, that is, an almost 10^{35}-fold (!) expansion. Thus, after 86 or fewer doublings, four cloned populations acquired at least one swa-resistant mutant per 500,000 infected cells (the number of cells challenged with swa), two were questionable and three remained incompetent.

Supplementary Fig S1 online (top) shows that there was no significant difference in the glycosylation status of the PrP^{Sc} of the nine clones we examined before swa treatment, and supplementary Fig S2 online (bottom) shows that after swa treatment clones #5–#9 had no detectable PrP^{Sc}, in line with the absence of swa competence documented in Table 1. Moreover, there was no

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Table 1 | Acquisition of swa competence by various cloned prion populations

#	Prion clone	Doublings				Incubation time* (sick/inoculated)	Sample injected†
		31	53	86	116		
1	8C4	+				240 ± 4 (4/4)	Conc.cond.medium
2	3C6	+				236 ± 22 (4/4)	Conc.cond.medium
3	8A8	−	+			ND	NA
4	3B12	−	−	+		251 ± 12 (4/4)	Conc.cond.medium
5	8H6	−	−	(+) (very low level)		ND	NA
6	8B4	−	−	(+) (questionable)		ND	NA
7	0.3-B2	−	−	−	ND	251 ± 10 (4/4)	Conc.cond.medium
8	0.3-B3	−	−	−	ND	221 ± 14 (5/6)	Conc.cond.medium
9	cdB8				−	190 ± 6 (6/6)	Cell lysate

Prions from PK1[22 L] cell supernatant (#1–8; described in Li *et al* (2010)) and from brain[22 L] homogenate (#9, described in Methods) were cloned by end point dilution in PK1 cells, propagated for the indicated number of doublings in the absence of swainsonine (swa) and then challenged with swa. The data for clones #1–3, at 31 and 53 doublings, are from Li *et al* (2010). + denotes emergence of swa-competent populations. 'Very low level', very low levels of swa-resistant prions were recovered after exposure to swa. − denotes swa-incompetent prions. *Incubation time, time elapsed between inoculation and terminal disease. †Mice were inoculated intracerebrally (i.c.) with 15 μl of 100 × concentrated conditioned medium (conc.cond.medium) from cells infected with the prion clones #1–8, collected after the designated number of doublings, while for clone #9 30 μl of cell lysate (5×10^7 cells/ml) was injected i.c. into C57BL/6 mice, which were culled when they exhibited definitive disease symptoms. NA, not applicable; ND, not done.

difference in PrP^{Sc} levels between swa-resistant clones grown in swa (S10) and swa-sensitive clones (C10) grown in the absence of the drug (supplementary Fig S2 online).

We determined the proportion of swa-resistant prions in PK1[22L]$wp_{(10)}$ (PK1 cells infected with brain[22L], after ten 1:10 splits) and in cloned populations #1, #7 and #9 (8C4, 0.3-B2 and cdB8) by the frequency assay (Li *et al*, 2010). As seen from Table 2, the proportion of swa-resistant prions in the populations ranged from 1.2% for PK1[22L]$wp_{(10)}$ to less than 0.0057% for #9. Because the cloned populations were initially swa incompetent (Li *et al*, 2010), those that became competent must have acquired swa-resistant prions by mutation. The low proportion of swa-resistant prions in #7 (0.3-B2) and #9 (cdB8) populations might account for our failure to obtain swa resistance when challenging these populations with swa.

Are these swa-incompetent clones permanently incompetent? To explore this question, mice were inoculated intracerebrally with concentrated conditioned medium from clones #1, 2, 4, 7 and 8 and cell lysate from clone #9 (Table 1) into four or six C57BL/6 mice each. In all cases, mice succumbed to scrapie-like disease after 190–250 days with 100% attack rates (4/4, 6/6), with the exception of clone #8, where 5/6 mice succumbed. The brain homogenates, as judged by the CPA, were indistinguishable from those injected with standard 22L-infected brain, that is, they were fully swa resistant and R33 competent (Fig 3), showing that, surprisingly, even the swa-incompetent prions had given rise to swa-resistant populations after propagating in the brain.

Next, PK1 cells were infected with 10^{-4} brain homogenate from mice inoculated with clones #7 and #9 (to yield brain[PK1(22L)$_{\#7}$] and brain[PK1(22L)$_{\#9}$], respectively) and as control with brain[22L] homogenate, and propagated for nine 1:20 splits (39 doublings) in the absence of swa. The prions in the conditioned media were assayed early on (P0) or after nine 1:20 splits (P9). In all cases the prions were largely swa resistant at P0 and swa sensitive at P9; however, if propagated in the presence of swa for five 1:20 splits (22 doublings), they were swa resistant (Fig 4). Thus, cell-adapted, swa-incompetent prions gave rise to swa-resistant prions after propagation in brain and, when again passaged in PK1 cells, became swa sensitive while retaining swa competence, showing that mutability had been restored.

DISCUSSION

Swa is an inhibitor of α-mannosidase II and causes misglycosylation of *N*-linked glycans, including those on PrP^{Sc}. We speculate that swa resistance reflects a conformational change, or 'mutation' of PrP^{Sc}, and is not due to its misglycosylation, because resistance persisted for many doublings even after propagation in the absence of the drug had restored normal glycosylation (Li *et al*, 2010). The conjectured conformational change we are considering is presumably minor because it did not result in a physicochemical alteration we could measure, and involves a low activation energy barrier, because it was reversed after five splits in the absence of swa (Li *et al*, 2010). How could such 'mutations' come about? It is likely that the conformational stability of small PrP^{Sc} aggregates is lower than that of larger ones (Weissmann *et al*, 2011), so that a moderate conformational change of a small seed, caused by thermal fluctuations, could come about and be 'fixed' as PrP (prion protein) accretion converted it into a larger, stable aggregate (Fig 1B).

Fig 1A summarizes the experiments described above. Brain-derived 22L prion populations consist almost entirely of swa-resistant prions (Table 3; Fig 1Aa); when transferred to PK1 cells (Fig 1Ab), the proportion of swa-resistant prions drops to about 60% after 11 doublings and 6% after 36 doublings (Table 3). These findings indicate that the brain-derived prion population contained a low level of swa-sensitive prions, which, as shown earlier (Fig 2C in Li *et al* (2010), have a selective advantage over their swa-resistant counterparts when propagated in PK1 cells and ultimately dominate the population (Fig 1Ac). When PK1 cells containing such swa-sensitive prions were propagated

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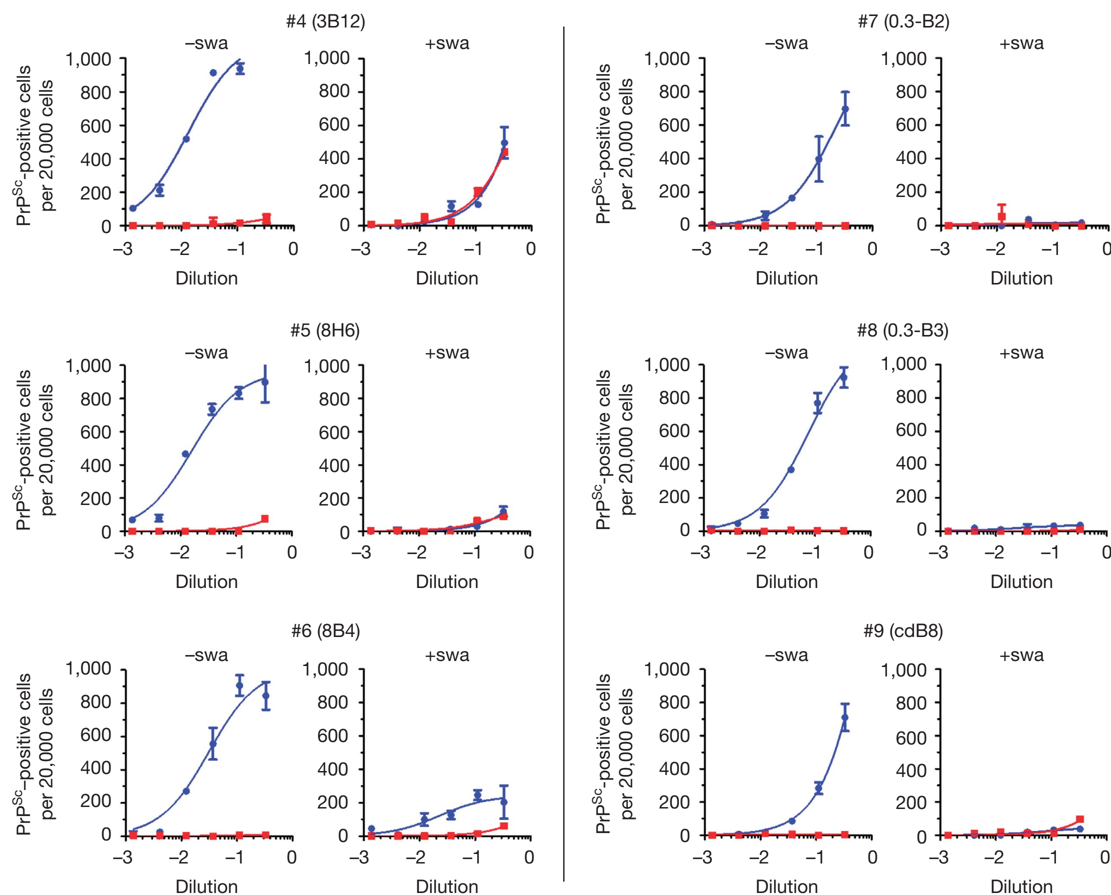

Fig 2 | Swa competence of various 22L prion clones. PK1 cells containing clones #4–8 after altogether 86 doublings, and #9 after 116 doublings, were propagated for five 1:20 splits in the presence or absence of 2 μg/ml swa, and the 100 × concentrated conditioned medium was subjected to the SSCA (standard scrapie cell assay) on PK1 cells in the absence (blue) or presence (red) of 2 μg/ml swa. The prions of clones #7, #8 and #9 were wiped out after propagation in swa, and were therefore swa incompetent. Clone #4 (3B12) prions became fully swa resistant, whereas clone #5 (8H6) prions persisted at low levels in swa and were swa resistant. Clone #6 (8B4) prions survived at a low level but showed only marginal swa resistance. The analysis of clones #1–3 is reported in Li *et al* (2010). Each point is the average of triplicate measurements; the bars indicate standard deviations. swa, swainsonine.

in the presence of swa, the swa-resistant prion minority rapidly became dominant (Fig 1Ad). Development of quinacrine resistance by murine prions in mouse brain has been reported by Ghaemmaghami *et al* (2009). Shorter and his colleagues described inhibition of yeast prion Sup35 propagation by the small molecules EGCG and 4,5-dianilinophthalimide (DAPH12), and selection of variants resistant to these compounds (Roberts *et al*, 2009; Shorter, 2010).

When prions from swa-sensitive populations were cloned by end point dilution into PK1 cells (Fig 1Ae), clones with distinct phenotypes with regard to mutability were obtained (I–III). All clones were initially swa sensitive and incompetent; that is, when challenged with swa the infected cells were cured of prions (Fig 1Af). Type I clones (represented by #1 and #2) acquired a low level of swa-resistant prions within ~30 doublings (Fig 1Ag), and while remaining swa sensitive became swa competent (Fig 1Ah). Type II clones (such as #3 and #4) became swa competent within about 50–90 doublings, whereas type III clones (#9) failed to do so even after 116 doublings. We interpret this as indicating that the three types of clone acquired mutations imparting swa resistance after distinctly different periods of propagation, perhaps due to different stabilities of the cognate PrP^{Sc}. Propagation of the three types of clone in mouse brain resulted in swa-resistant 22L prions (Fig 1Ai). Because the type III prion population was swa incompetent before inoculation, we assume that it either contained swa-resistant mutants at a level undetectable by our method of competence determination or that mutability increased as the prions adapted to the brain. As shown in Fig 1Ab′–c′, the brain-derived swa-resistant prion population, when transferred to PK1 cells, again became swa sensitive while remaining swa competent. The important conclusion is that PrP^{Sc} can adopt not only swa-sensitive and swa-resistant conformations, but also conformations that show different degrees of stability, as reflected by their mutation frequencies. Thus, mutability might be viewed as a further phenotype contributing to the heterogeneity of prion populations postulated

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Table 2 | Frequency of swa-resistant prions in various populations

Sample	swa	Pos/total wells	Confirmed pos/total wells	$m = \ln[1/1 - P_{pos}]$*	Cells/well	Frequency = m/(cells/well)	F_{+swa}/F_{-swa}
PK1[22 L]$wp_{(10)}$†	−	11/267 = 0.041		0.042	5	0.0084	0.012
	+	36/267 = 0.135	27‡/267 = 0.10	0.105	1,000	0.0001	
#1 8C4	−	8/267 = 0.030		0.030	5	0.0060	0.0013
	+	2/267 = 0.0075	2/267 = 0.0075	0.0075	1,000	0.0000075	
#7 0.3-B2	−	9/267 = 0.034		0.033	5	0.0066	0.00060
	+	9/890 = 0.010	7.5§/890 = 0.0084	0.0084	2,000	0.0000042	
#9 cdB8	−	12/267 = 0.045		0.046	5	0.0088	< 0.000057
	+	6/890 = 0.0067	< 1/890 = < 0.001	< 0.001	2,000	< 0.0000005	

PK1 cells were infected with concentrated, conditioned medium from the sample indicated, in either the absence or presence of swainsonine (swa), and pools of 5 cells (in absence of swa) and 1,000 or 2,000 cells (in presence of swa) were distributed into the indicated number of wells of 96-well plates. After reaching confluence, the cells were subjected to five 1:10 splits and analysed by PK-ELISA. A well was considered positive (pos) if the number of spots exceeded the average spot number +5 s.d.s given by uninfected wells. Any well that scored positive was assumed to have initially contained one or more prions. Because some wells were borderline positive, all 'swa-positive' wells of #1 and #9, and 4 of 36 and 6 of 9 'positive' wells in the case of PK1[22 L]$wp_{(10)}$ and #7, respectively, were re-examined by expanding the cells and testing the secreted prions for swa resistance by the SSCA (standard scrapie cell assay) yielding 'confirmed positives/total'. The percentage swa-resistant prions was calculated by the Poisson equation, taking into account the pool sizes. *From the Poisson equation $P_{(0)} = e^{-m}$ we derive $P_{pos} = P_{(\geq 1)} = 1 - P_{(0)} = 1 - e^{-m}$. Hence $m = \ln[1/(1 - P_{pos})]$. $P_{(0)}$, probability of an uninfected well; $P_{pos} = P_{(\geq 1)}$, probability of a positive well; m, average number of infected cells/well. †Passaged for ten 1:10 splits (33 doublings). ‡Three of four tested were true positives. §Five of six tested were true positives.

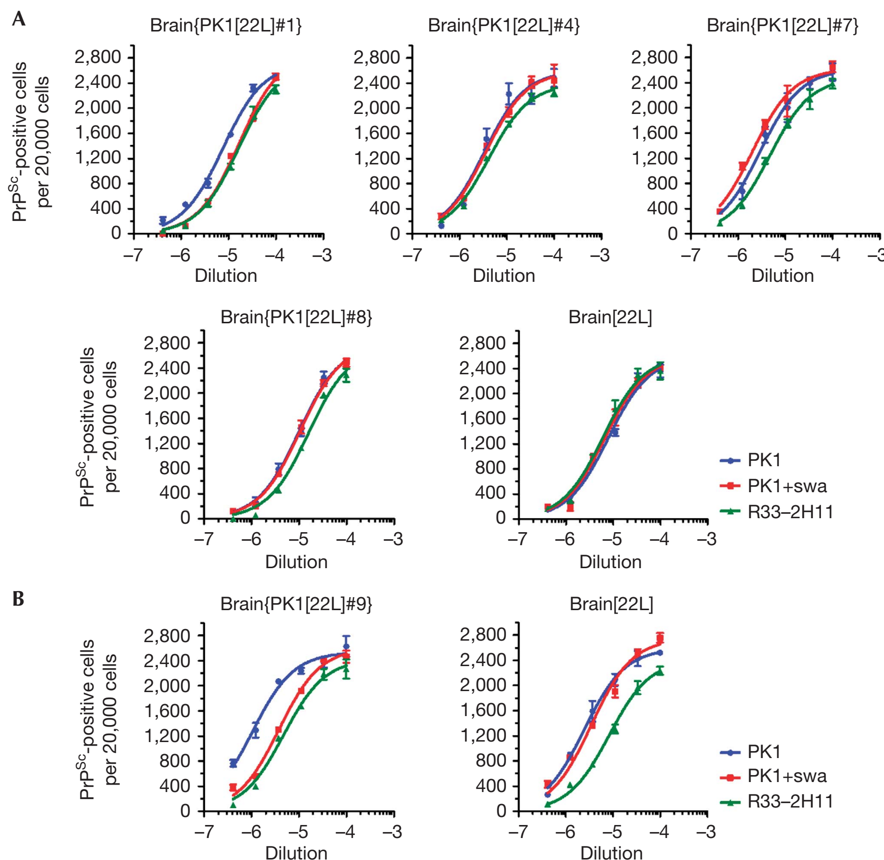

Fig 3 | SSCA of homogenates of brains infected with various PK1[22L] prion clones. Concentrated conditioned medium from PK1[22L]#1, -#4, -#7 and -#8 or lysate from PK1[22L]#9 was inoculated intracerebrally into C57BL/6 mice, as detailed in Table 1, to yield brain{PK1[22L]#1}, brain{PK1[22L]#4}, brain{PK1[22L]#7}, brain{PK1[22L]#8} and brain{PK1[22L]#9}. The brain homogenates were subjected to the SSCA in the absence (blue) or presence (red) of 2 μg/ml swa. **A** and **B** were assayed in separate experiments, in parallel with brain[22L] homogenate as reference. All samples were equally swa resistant and R33-2H11 competent. Each point is the average of triplicate measurements; the bars indicate standard deviations. SSCA, standard scrapie cell assay; swa, swainsonine.

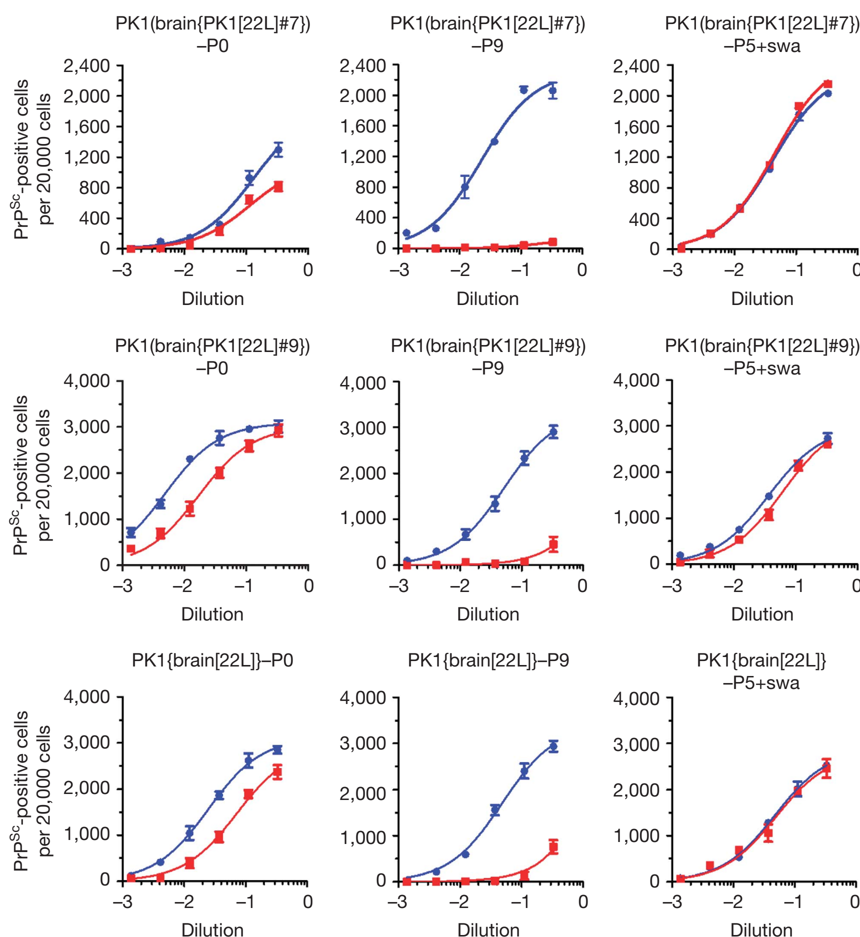

Fig 4 | SSCA assay of 22L prions cloned in PK1 cells, passaged through brain and returned to PK1 cells. PK1 cells were infected with the homogenates of brain{PK1[22L]#7}, brain{PK1[22L]#9} and brain[22L]; the conditioned medium of these cells was analysed at passage 0 (P0), after nine 1:20 splits in the absence of swa (P9) or after five 1:20 splits in the presence of 2 μg/ml swa (P5 + swa). In all cases, at P0, the prions secreted by the freshly infected cells were largely swa resistant, after nine splits they had become swa sensitive, but after five splits in the presence of swa they retained swa resistance. Samples were assayed in the absence (blue) or presence (red) of 2 μg/ml swa. Each point is the average of triplicate measurements; the bars indicate standard deviations. SSCA, standard scrapie cell assay; swa, swainsonine.

by the 'quasi-species' hypothesis (Eigen, 1996; Collinge & Clarke, 2007; Li *et al*, 2010; Shorter, 2010).

METHODS

Cells. PK1 and R33-2H11 cells (Mahal *et al*, 2010) were cultured in Opti-MEM (Invitrogen) containing 5% or 9% bovine growth serum (BGS (OBGS); Hyclone, Logan, UT; there was no difference in growth rate of cells or outcome of the SSCA with 5% or 9% BGS), 90 units/ml penicillin and 90 μg/ml streptomycin (Invitrogen). Cells were maintained by splitting 1:10. Both cell lines contain the *Prnp-a* allele (C. Baker, S. Browning and C.W., unpublished results), as do C57BL/6 mice (Westaway *et al*, 1994).

Preparation of cell lysates. Cells (5×10^7/ml in PBS) were frozen and thawed four times in liquid nitrogen and passed through a 28-gauge needle.

Preparation of brain homogenates. The 22L strain, biologically cloned in mice by two successive end point dilutions, was obtained from I. McConnell and R.M. Barron (TSE Resource Centre, Compton, Newbury, UK) and propagated in C57BL/6 mice (Charles River Laboratories). Frozen brains were homogenized for 10 s in PBS (9 ml/g) using a hand-held Ultramax T18 basic homogenizer (IKA Works, Bloomington, NC) at 20,000–25,000 r.p.m. Single frozen brains were homogenized using a ribolyser (FastPrep FP120, Bio 101, Thermo Electron, Thermo Fischer Scientific) with ZrO 0.8- to 1-mm beads (7305–000010; Glen Mills) at maximum speed (setting 6.5) for 15 s in Fast Prep tubes (MP Biomedicals). Homogenates were stored in small aliquots at −80 °C. Thawed homogenates were re-homogenized by passing through a 28-gauge needle; they were not centrifuged at any stage. The titre, determined by mouse bioassay, in LD_{50} units per gram brain, was $10^{8.3}$ for 22L.

Standard scrapie cell assay (SSCA). The assay has been described in detail in Mahal *et al* (2010). In short, 5,000 cells in the wells of 96-well plates were exposed to a serial dilution of the prion-containing sample for 4 days, split three times 1:10 after reaching confluence. A total of 20,000 cells were dried onto the membranes

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Table 3 | Frequency of swa-resistant prions in various populations

Cells/well**	swa	Brain[22 L] hom.		PK1[22 L]P0* (cond.med.)		PK1[22 L]P8* (cond.med.)		PK1[22 L]cdB8 (cond.med.)	
		Pos/48	Swa res. (%)	Pos/48	Swa res. (%)	Pos/48	Swa res. (%)	Pos/48	Swa res. (%)
1,000	–	46		47		23	4.3	39	0
	+	46		48		1		0	
500	–	32	100	47		10	10	21	0
	+	33		47		1		0	
250	–	15	93	48		6		10	0
	+	14		48		0		0	
125	–	8		47		3		11	0
	+	11		44		0		0	
62	–	4		44		0		5	
	+	9		26		0		0	
31	–	6		25	64	0		3	
	+	9		16		0		0	
15	–	2		**12**	75	0		1	
	+	2		**9**		0		0	
Average ± s.d. (%)		96.5 ± 5		69.5 ± 7.8		7.1 ± 4		0 (<2.6)	

PK1 cells were infected with dilutions of the 100 × concentrated conditioned medium: 1:13 for PK1[22 L]$_{P(0)}$), 1:166 for PK1[22 L]$_{P(8)}$ and 1:3 for PK1[22 L]cdB8, or with a 3×10^{-6} dilution of brain[22 L] homogenate, all in either the presence or absence of 2 μg/ml swa. The number of cells indicated (**), plus uninfected PK1 cells required to bring the total up to 1,000 cells were placed in 48 wells. Because we did not know what frequencies to expect, we used a wide range of cells/well. The cells were grown to confluence, split 1:10 five times and PrP^{Sc}-positive (pos) wells were identified as described in the legend to Table 2. Only the samples that gave between 10 and 40 PrP^{Sc}-positive wells per 48 wells in the absence of swa were evaluated to avoid excessive fluctuation at the lower values and saturation at the higher ones. The values in the shaded fields were not included in the calculation of averages.
*PK1 cells were infected with 22 L-infected brain homogenate (brain[22 L]), expanded from one well of a 6-well plate to ten 15-cm dishes (P(0)) and propagated for 11 or 36 doublings to yield PK1[22 L]$_{P(0)}$ and PK1[22 L]$_{P(8)}$, respectively. Secreted prions were concentrated 100 × from conditioned medium (cond.med.) by 2 h centrifugation at 100,000 × *g*. Hom, homogenate; Pos/48, PrP^{Sc}-positive wells per 48 wells; res., resistant; swa, swainsonine.

of 96-well filter plates, treated with PK and denatured with guanidinium thiocyanate; PrP^{Sc}-positive cells were revealed by ELISA with PrP antibody D18 and counted using Zeiss KS Elispot imaging equipment, and, more recently, the Bioreader 500-Eb (Biosys).

PK-ELISA assay. To determine the proportion of PrP^{Sc}-containing cells in a population, a serial 1:2 dilution from 20,000 to 625 cells was placed in the wells of a 96-well plate and subjected to the ELISA described above.

Determination of swa competence. PK1[22L] clones were expanded to 95% confluence in a 15-cm plate in OBGS, split 1:20 into two 15-cm dishes and the cells were passaged for five 1:20 splits in the absence or presence of 2 μg/ml swa (11.55 μM). Conditioned medium from ten 15-cm dishes of cells was centrifuged for 2 h at 35,000 r.p.m. in a TI45 rotor. Pellets were gently rinsed with PBS, dissolved in 1/100 the original volume of OBGS and subjected to the SSCA on PK1 cells in the presence or absence of swa.

End point dilution cloning of brain[22L] homogenate in PK1 cells. Five thousand PK1 cells were exposed to 3×10^{-5}, 10^{-5}, 3×10^{-6} and 10^{-6} dilutions of 22L-infected brain homogenate from *tga20* mice (Fischer *et al*, 1996; Karapetyan *et al*, 2009). After 4 days, the cells from each dilution were distributed into three 96-well plates at an average of 0.5 cells/well. After 1 week, wells containing a single colony were marked, grown to confluence for another 7 days and subjected to the PK-ELISA assay. Only the 10^{-5} dilution gave rise to PrP^{Sc}-positive colonies. At this dilution, 27% of wells contained a single-cell colony of which two were PrP^{Sc} positive. Clones cdB8 and cdE4 comprised 20% and 27% PrP^{Sc}-positive cells, respectively, as determined by the PK-ELISA assay. The clones were expanded from wells of 96-well plates to twenty 15-cm dishes (about 27 doublings) and frozen down.

Frequency assay. This assay allows the determination of the proportion of swa-resistant prions in a population. The procedure outlined below is designed for populations in which the range of frequencies is unknown; if the range is known, a protocol such as that shown in Table 2 might be adopted. PK1 cells were infected with appropriately diluted 22L-infected brain homogenate or concentrated conditioned medium in the presence or absence of 2 μg/ml swa. Pools of 1,000, 500, 250, 125, 62 or 31 cells infected in the absence or presence of swa were dispensed into 48 wells each of a 96-well plate, and uninfected PK1 cells were added to bring the total cells/well to 1,000. Each plate contained 12 wells of uninfected cells for background determination. After reaching confluence, the cells of each well were split 1:10. After altogether five splits, the cells were grown to confluence and 20,000 cells were filtered off onto 96-well filter plates and processed as described for the SSCA. The plates with the remaining cells were retained for subsequent validation. A well was considered positive if the number of spots exceeded

the average spot number given by uninfected wells +5 standard deviations. Positive wells, or a sample of positive wells from a particular set, were validated by expanding the cells to ten 15-cm dishes and assaying a serial dilution of a 100× concentrated supernatant on PK1 cells in the presence or absence of swa.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: J.L. performed most of the experiments, S.P.M. and C.A.D. isolated, characterized and propagated clone cdB8 (#9), C.W. supervised the study and wrote the manuscript.

CONFLICT OF INTEREST
The authors have no conflict of interests.

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