Investigating CRISPR/Cas9 gene drive for production of disease-preventing prion gene alleles

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Abstract

Prion diseases are a group of fatal neurodegenerative disorders that includes chronic wasting disease, which affects cervids and is highly transmissible. Given that chronic wasting disease prevalence exceeds 30% in some endemic areas of North America, and that eventual transmission to other mammalian species, potentially including humans, cannot be ruled out, novel control strategies beyond population management via hunting and/or culling must be investigated. Prion diseases depend upon post-translational conversion of the cellular prion protein, encoded by the Prnp gene, into a disease-associated conformation; ablation of cellular prion protein expression, which is generally well-tolerated, eliminates prion disease susceptibility entirely. Inspired by demonstrations of gene drive in caged mosquito species, we aimed to test whether a CRISPR/Cas9-based gene drive mechanism could, in principle, promote the spread of a null Prnp allele among mammalian populations. First, we showed that transient co-expression of Cas9 and Prnp-directed guide RNAs in RK13 cells generates indels within the Prnp open-reading frame, indicating that repair of Cas9-induced double-strand breaks by non-homologous end-joining had taken place. Second, we integrated a ~1.2 kb donor DNA sequence into the Prnp open-reading frame in N2a cells by homology-directed repair following Cas9-induced cleavages and confirmed that integration occurred precisely in most cases. Third, we demonstrated that electroporation of Cas9/guide RNA ribonucleoprotein complexes into fertilised mouse oocytes resulted in pups with a variety of disruptions to the Prnp open reading frame, with a new coisogenic line of Prnp-null mice obtained as part of this work. However, a technical challenge in obtaining expression of Cas9 in the male germline prevented implementation of a complete gene drive mechanism in mice.

Introduction

Prion diseases are uniformly fatal, neurodegenerative disorders of mammals. One such disorder is chronic wasting disease (CWD), which affects cervids and is highly transmissible. CWD was first identified in Colorado in the 1960s and, as of 2019, had spread to 26 U.S. states and three Canadian provinces, with cases also detected in several European countries and in South
Korea [1]. With disease prevalence in wild cervids exceeding 30% in some endemic areas of North America [2], CWD presents significant challenges for the deer hunting and farming industries. Furthermore, while prion diseases are generally subject to a “species barrier” in terms of transmission [3, 4], this barrier is not absolute, and concerns remain that CWD could spread to other mammalian species [5], perhaps even to humans [6]. Without effective prophylactics or therapeutics, control strategies for CWD are currently restricted, with limited success, to population management via hunting and/or culling [7]. Several vaccination trials have been performed [8–11], with the ultimate aim of protecting farmed cervids, but one of the most recent studies reported that the vaccine candidate actually accelerated CWD onset [11]. It is therefore imperative to seek additional approaches to deal with CWD and other prion diseases that may emerge in animal populations.

The cellular prion protein (PrP<sup>C</sup>), encoded by the Prnp gene, is a glycoprotein that normally resides on the surface of cells [12], particularly neurons [13]. A key hallmark of prion disease is the template-mediated conversion of PrP<sup>C</sup> substrate into abnormal, partially protease-resistant conformations often referred to as PrP<sup>Sc</sup> [14–16]. Thus, partial reduction of PrP<sup>C</sup> expression in Prnp hemizygotes [17] or use of Prnp mRNA-directed antisense oligonucleotides impedes prions infections [18, 19], while complete elimination of PrP<sup>C</sup> expression prevents prion infections entirely [20, 21]. Importantly, no severe phenotypes have been detected in genetically engineered PrP<sup>Sc</sup>-null animals [22–26] or in goats with a naturally occurring premature stop codon within the Prnp open-reading frame (ORF) that ablates PrP<sup>C</sup> expression [27]. The only consistently observed phenotype of PrP<sup>Sc</sup>-null animals is a relatively late-onset peripheral neuropathy with mild phenotypic consequences [28–30]. Therefore, we asked whether recent advances in gene editing technologies could be exploited to eliminate PrP<sup>C</sup> expression and, consequently, impart prion disease resistance into animal populations. Given the absolute requirement for PrP<sup>C</sup> substrate, such an approach would have the crucial advantage of conferring resistance to all prion strains unlike methodologies which target misfolded PrP forms. In principle, Prnp could be knocked out via gene editing in a small number of captive cervids, which could then be used in breeding programs. However, a gene drive mechanism would theoretically be capable of spreading prion disease resistance much faster and could also be effective in wild cervid populations. Gene drives are “selfish” molecular mechanisms that promote inheritance of a DNA sequence at super-Mendelian frequencies. Such mechanisms exist naturally (reviewed in [31]) and include transposable elements, meiotic drivers, and mating type switching in yeast. Recent years, however, have seen renewed interest in synthetic gene drive systems that take advantage of CRISPR/Cas9 technology. CRISPR/Cas9-based gene drives have proven effective in laboratory settings, particularly in malaria vector mosquito species [32–35], and are being considered seriously for the eradication of invasive species in countries such as New Zealand [36].

Here, in a controlled laboratory setting, we investigated whether a CRISPR/Cas9-based gene drive could, in principle, be used to promote the spread of a null Prnp allele. We succeeded in generating reagents able to modify the murine Prnp locus in cultured cells via homology-directed repair (HDR) of Cas9-induced double-strand breaks (DSBs) and describe an issue pertaining to germline expression of Cas9 in mice.

Results

Design of a CRISPR/Cas9-based gene drive mechanism to spread prion disease resistance

The gene drive mechanism we designed is outlined in Fig 1. Briefly, we planned to create a donor DNA cassette containing Cas9 and guide RNA (gRNA) coding sequences under the
control of germline-specific and ubiquitous promoters (e.g., the U6 RNA polymerase III promoter [37]), respectively, as well as a modified GFP reporter transgene (explained in more detail later in the manuscript). Prnp-specific homology arms of ~800 bp would flank these sequences; homology arms of this length are recommended when using double-stranded DNA donors [38]. Ribonucleoprotein (RNP) complexes of Cas9 and a Prnp-specific gRNA would be delivered to generate a DSB within the Prnp ORF, at which point, in the presence of the donor DNA cassette, HDR would result in integration of the donor sequence. This process would create a mobile null Prnp allele able to convert the wild type (WT) allele on the sister chromosome in the germline.

A first step was to select suitable gRNA spacer sequences for targeting Prnp. The initial codons of the Prnp ORF were avoided in case Cas9 cleavage disrupted the splice acceptor site of the protein-coding exon, which can result in chimeric transcripts derived from Prnp and extending into the neighbouring gene Prnd; this process leads to ectopic expression of Doppel (the protein product of Prnd) in the brain, where it is neurotoxic [39]. Therefore, we used a
gRNA design algorithm (no longer available but previously found at http://crispr.mit.edu) to scan codons 23–50 of murine Prnp, noting that residues 1–22 comprise the N-terminal signal peptide of PrpC that is absent from the mature protein (residues 23–231 in mice). The top scoring sequences were used to generate three slightly different gRNA spacers for testing (S1 Fig, panels A and B). The chosen spacers are a few codons downstream of the sequence used by Mehrabian et al. (2014) to knock out PrpC expression in cultured cells [40].

Selected Prnp gRNAs induce Cas9-mediated cleavage within the Prnp ORF

Having selected Prnp-specific spacer sequences, we prepared RNP complexes consisting of recombinant Cas9 (recCas9) and tracrRNA/crRNA gRNA duplexes. After confirming that recCas9/Prnp gRNA–1 RNP complexes induced cleavage of a Prnp expression plasmid in vitro (Fig 2A), we cloned the spacer sequences separately into the eSpCas9(1.1) vector [41], which is capable of expressing an enhanced-specificity Cas9 (i.e., eCas9) and a single gRNA under control of the CMV and U6 promoters, respectively. Next, we transiently transfected the modified eSpCas9(1.1) vectors into RK13 rabbit kidney epithelial cells stably expressing WT murine PrpC (clone WT-5); expression of the endogenous rabbit PrpC is undetectable in the RK13 cell line [42]. Forty-eight hours later, we used fluorescence-activated cell sorting (FACS) in an attempt to identify cells with reduced PrpC expression due to non-homologous end-joining (NHEJ)-mediated disruptions of the murine Prnp ORF. However, expression of Prnp-directed gRNAs did not result in obvious reductions in PrpC signal intensities when compared with the empty vector control condition. We therefore selected the cells in approximately the lowest decile of PrpC signals for each sample (S2 Fig) and performed T7E1 mismatch cleavage assays on the pooled DNA. Faint bands indicative of Prnp ORF disruptions were detected for all three gRNAs (Fig 2B), demonstrating that NHEJ events induced by Cas9 cleavage activity had occurred, albeit most likely at low frequency. Equivalent experiments were attempted in the

Fig 2. Selected Prnp gRNAs induce Cas9-mediated cleavage within the Prnp ORF. (A) Agarose gel image showing that recCas9 cleaves a half-genomic Prnp expression construct (MoPrP.Xho.wt) when Prnp gRNA–1 is present. A cleavage product of the expected size is indicated by the red arrow. (B) Agarose gel images showing the products of T7E1 mismatch cleavage assay reactions for DNA samples obtained from untransfected WT-5 RK13 cells (Cas9-negative) or cells transfected with eSpCas9(1.1) expression plasmids containing Prnp gRNA–1, –2 or –3, or no gRNA. In the absence of Prnp ORF disruptions, a PCR product of ~900 bp was expected. The faint bands appearing at ~400 and 500 bp only when Prnp gRNAs were expressed indicate that indels resulting from NHEJ events were present.

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MDB (mule deer brain) cell line [43] with gRNAs directed against the endogenous cervid Prnp, but low transfection efficiencies prevented further progress in this regard.

Integration of a DNA cassette into the Prnp locus of murine neuroblastoma cells by HDR of Cas9-induced DSBs

Thus far, we have shown that the selected Prnp gRNAs direct Cas9 to cleave within the Prnp ORF both in vitro and in cell culture. The next step towards the goal of replacing endogenous Prnp with a mobile null allele capable of gene drive was to prepare a donor plasmid vector containing a simplified version of the DNA cassette shown in Fig 1, specifically the modified GFP reporter gene flanked by ~800 bp Prnp homology arms (Fig 3A). The reporter gene consisted of the GFP coding sequence (minus its start codon) fused to codons 230–254 of murine Prnp, which encode the PrP\textsuperscript{C} C-terminal signal peptide, thereby generating a transgene that we termed Gfp-GPI. HDR-mediated integration of the donor DNA cassette should result in expression of GFP driven by the Prnp regulatory elements, with the N- and C-terminal signal peptides of PrP\textsuperscript{C} attached, enabling transit into the secretory pathway and expression at the cell surface via a glycophaspatidylinositol (GPI) anchor attachment, as has been demonstrated previously [44].

N2a murine neuroblastoma cells were co-transfected with the donor vector and eSpCas9 (1.1) expression vectors containing Prnp gRNAs. Unfortunately, we were unable to detect cells expressing the reporter using fluorescence microscopy to examine bulk cell populations. Nonetheless, integration of the Gfp-GPI transgene did occur, at least in some cells, because 3’ junction PCR analysis of pooled DNA samples collected 3 days after transfection resulted in

![Diagram of the donor vector generated for HDR experiments. Prnp homology arms flank a promoter-less reporter gene consisting of the Gfp ORF fused to the 3’ end of the Prnp ORF that would normally encode residues 230–254 of PrP\textsuperscript{C}.](https://doi.org/10.1371/journal.pone.0269342.g003)
bands of the expected size only when \( \text{Prnp} \) gRNAs were present (Fig 3B). A follow-up 5' junction PCR analysis for \( \text{Prnp} \) gRNA–1, the best performing of the three gRNAs in the initial experiment, also resulted in bands indicative of donor DNA integration (Fig 3C). Providing more time after transfection for editing to occur (6 days) appeared to result in more intense bands in the diagnostic PCR for the 5' and 3' junction fragments (Fig 3D), although a side-by-side numerical comparison of the two conditions was not performed.

Even in the presence of a donor DNA cassette, Cas9-induced DSBs may still be repaired by NHEJ on some occasions. Indels derived from these NHEJ events could make the \( \text{Prnp} \) alleles permanently resistant to the Cas9/gRNA combination. Alternatively, the presence of small indels may still permit subsequent HDR events to occur; depending on the nature of the repair, the reporter transgene may not function effectively or the CRISPR protospacer sequence within the endogenous \( \text{Prnp} \) may not be split as intended, potentially rendering the null \( \text{Prnp} \) allele itself vulnerable to new rounds of Cas9-mediated cleavage. Because of these issues we proceeded to check whether integration of the donor DNA cassette had occurred precisely in the N2a cells. The junction PCR products shown in Fig 3C were gel-purified, cloned into pCr2.1-TOPO, and transformed into \textit{E. coli}. Twelve clones in total were selected, six for each junction. Diagnostic restriction digests confirmed the presence of an insert of the correct size in 5/6 cases for the 3' junction and 6/6 for the 5' junction (S3 Fig, panel A). Sanger sequencing of four of the 5' junction clones and three of the 3' junction clones with normal restriction digest profiles showed that the expected sequence was present with no indels detected around the junction sites (S3 Fig, panels B and C). The 3' junction clone with the abnormal restriction digest profile (#3) was also sequenced, but the data were hard to interpret, with tracts of the expected sequence identified together with other unknown sequences. Nonetheless, considered as a whole, these data show that we succeeded in integrating a ~1.2 kb DNA sequence (the \( \text{Gfp-GPI} \) transgene) into the \( \text{Prnp} \) ORF of N2a cells and that this integration occurred precisely in the majority of cases.

**Generation of CRISPR/Cas9-induced disruptions of the \( \text{Prnp} \) ORF in mice**

Following the successful demonstration of \( \text{Prnp} \) editing in cell culture, we tested the effectiveness of our chosen \( \text{Prnp} \) gRNAs \textit{in vivo}. The results of these experiments are summarized in Table 1. Two attempts at microinjecting recCas9/\( \text{Prnp} \) gRNA–3 RNP complexes (tracrRNA/crRNA gRNA duplex format) into fertilized FVB/NJ mouse oocytes were unsuccessful; sequencing of 31 viable pups revealed no signs of \( \text{Prnp} \) ORF disruptions. However, electroporation of RNP complexes, yielding a combined 18 live births from two experiments, led to the

| Experimental configuration                      | Allele yield* | Live mice obtained | Allele type                  | Inferred mechanism   |
|------------------------------------------------|---------------|--------------------|------------------------------|----------------------|
| Microinjection of oocytes with RNP complexes   | 0/31 (0/62)   | 31                 | N/A                         | N/A                 |
| Electroporation of oocytes with RNP complexes  | 3/18 (3/36)   | 18                 | 1 bp deletion (frameshift)   | Classical NHEJ       |
|                                                |               |                    | 6 bp deletion (in-frame)     | Alternative NHEJ (MMEJ) |
|                                                |               |                    | 21 bp deletion (in-frame)    | Alternative NHEJ (MMEJ) |
| Electroporation of oocytes with RNP complexes  | 1/9 (1/18)    | N/A (embryos harvested at 5.5 dpc) | 48 + 27 bp deletions (both in-frame) | 2 alternative NHEJ (MMEJ) events |

See Fig 4 and S4 Fig for more detailed results from these experiments. \( \text{Prnp} \) gRNA–3 was used in each case. New abbreviations: MMEJ, microhomology-mediated end-joining; dpc, days post coitus.

*The first value represents the modification frequency (i.e., successful modifications over the number of mice or mouse embryos screened). Numbers in parentheses are adjusted for the number of allele targets per diploid genome.

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identification of three founders with disruptions to one Prnp allele, as determined by Sanger sequencing (Fig 4A). Two founders (#34 and #36) had in-frame deletions (21 bp and 6 bp) in the vicinity of the expected Cas9 cleavage site; the presence of a disrupted Prnp allele in founder 34 was confirmed by T7E1 assay (Fig 4B). These in-frame deletions remove amino acids lying within the first of PrP<sup>C</sup>‘s two hexarepeats of the form GGN/SRYP. An additional founder (#33) had a frameshift mutation resulting from an insertion of 1 bp that was predicted to generate an abnormal sequence from codon 39 onwards with a new stop codon at position 78. Mice derived from each of the founders appeared phenotypically normal, although detailed characterization was not performed. Homozygous progeny were obtained for line 33 and a lack of PrP<sup>C</sup> expression in the brain was confirmed by capillary western analysis (Fig 4C). Furthermore, we obtained Sanger sequencing data from PCR amplicons containing three of the highest-scoring potential off-target sites for gRNA–3 that were identified by the gRNA design algorithm (S1 Fig, panel C), but no indels were detected for any of the three founders.

Because the deletion in founder 34 was quite large (21 bp), we wanted to determine whether such deletions would be commonplace. To simplify the process, Cas9/Prnp gRNA–3 RNP complexes were electroporated into fertilized oocytes as before, but this time the embryos were grown in vitro to 5.5 days post coitus (dpc) before being used for preparation of genomic DNA and subsequent direct sequencing of PCR products. Although sequencing data of suitable quality were obtained for nine embryos, only one had a disrupted Prnp allele. However, this allele had an unusual double deletion of 48 and 27 bp, with a short tract of Prnp sequence retained in between (S4 Fig).

Transgenic mice expressing Cas9 under control of the Prl3b1 promoter do not express Cas9 in the germline

Having created mice with Cas9-induced Prnp disruptions, we attempted to engineer mice capable of expressing Cas9 in the germline—an important step towards creating a fully mobile genetic element for gene drive. We searched for a male germline-specific promoter, because findings from an earlier study in mosquitoes suggested that expression in the female germline can lead to Cas9 persistence in the egg, causing NHEJ-induced indels that render the target gene resistant to the drive mechanism [32]. The gene Prl3b1 was chosen due to its reported activity in the germline of male mice [45], and transgenic (Tg) mice expressing eCas9 under control of the Prl3b1 promoter (Prl3b1-eCas9<sup>+/–</sup>) were generated. However, we were unable to detect expression of eCas9 in various male urogenital tissues of Prl3b1-eCas9<sup>+/–</sup> Tg mice derived from founder 4 (Fig 5A). Testes isolated from mice derived from other founders were also analysed, as were female reproductive tract tissues (ovaries) from the same mice, but no Cas9 expression was observed (Fig 5B). In addition to its reported activity in the male germline, Prl3b1 is expressed in the placenta [46]. We therefore crossed Prl3b1-eCas9<sup>+/–</sup> and WT mice and prepared homogenates of embryos extracted from 15 dpc pregnant females together with their respective placentas. Cas9 expression was detected in approximately half of the embryo-placenta homogenates (Fig 5C), which indicates that the transgene construct was functional and that the problem clearly lay with the promoter, which was not sufficiently active in the male germline for our purposes.

Discussion

Steps towards population-based eradication of prion disease

In this study, we embarked on a series of steps to develop a complete CRISPR/Cas9-based gene drive mechanism able to promote the spread of a null Prnp allele among a population of mice and hence confer resistance to prion infection. While this ultimate goal was not achieved,
we nonetheless showed that our selected Prnp gRNAs were active in mice and that we could modify the Prnp locus in N2a cells by introducing a donor DNA sequence via HDR of Cas9-induced DSBs. The reagents generated for the cell experiments could be repurposed to perform different edits to the Prnp locus, such as the introduction of disease-relevant mutations in order to study their effects. This approach would enable levels of the mutant Prnp to be controlled by the endogenous regulators of Prnp expression, in contrast to the more basic strategy of knocking out Prnp using CRISPR/Cas9 followed by random integration into the genome of a transgene construct or lentiviral vector containing the mutant Prnp.

The T7E1 assay data shown in Fig 2B suggest that a relatively low proportion of the RK13 cells contained disruptions to the murine Prnp ORF. In these experiments, the cells were harvested two days after transfection with the Cas9/gRNA expression vectors. As we discovered through subsequent experiments performed in N2a cells, harvesting the cells after a longer post-transfection time interval may have improved the cleavage efficiency. In addition, although improving gene editing performance in cultured cells was not the main focus of this study, co-treatment with cell cycle-regulating compounds would likely lead to further gains in Cas9 cleavage efficiency [47]. Similar experiments performed in MDB cells using gRNAs targeting the endogenous cervid Prnp were unsuccessful due to low transfection efficiencies; lentiviral transduction of CRISPR/Cas9 components might be a more effective approach for this cell line. Alternatively, experiments could be performed using an easily transfectable cell line (e.g., HEK293T) engineered to contain cervid Prnp.

Returning to the N2a cell experiments, integration of the donor DNA cassette into the genome typically occurred in a precise manner, with no errors detected around the junction sites in most cases. Although we expected precise integration of the donor cassette to result in expression of a GPI-anchored GFP reporter together with elimination of PrP<sup>C</sup> expression, we did not detect any green-fluorescent cells, perhaps because endogenous PrP<sup>C</sup> expression is relatively low in the N2a cell line (and expression of the reporter would be under control of the Prnp promoter) [48]. We did not assess whether the production of PrP<sup>C</sup> from edited alleles was eliminated, because N2a cells are reportedly at least tetraploid [49], on average, so disruption to one Prnp allele would be unlikely to reduce PrP<sup>C</sup> expression sufficiently for overt differences to be detected by immunofluorescence microscopy.

During testing of the selected Prnp gRNAs in vivo, we obtained a founder (#33) with a 1 bp insertion within the Prnp ORF resulting in a frameshift from codon 39 onwards, with a premature stop codon created at position 78. Although PrP<sup>C</sup> expression was not detected in homozygous progeny of founder 33 using the antibodies Sha31 (epitope 144–151; [50]) and 12B2 (epitope 88–92; [51]), further characterization is required to determine if the truncated protein encoded by the mutant Prnp allele is actually expressed and, if so, whether there are any gain-of-function effects. We would, however, expect line 33 to exhibit complete loss of normal PrP<sup>C</sup> function, given that only 16 of the remaining 38 unmodified codons are actually present in the mature protein (due to removal of the N-terminal signal peptide). We believe that these mice may be useful for other laboratories studying prion disorders, noting that a similar coisogenic

**Fig 4.** Generation of CRISPR/Cas9-induced disruptions of the Prnp ORF in mice. (A) Sanger sequencing chromatograms for mice derived from fertilized FVB/NJ oocytes that were electroporated with recCas9/Prnp gRNA–3 RNP complexes. The numbers below the automated base calls indicate the position in the sequencing read rather than the Prnp ORF. Changes to the PrP<sup>C</sup> amino acid sequence for the lines with disrupted Prnp are indicated in the bottom box. (B) Agarose gel image showing the products of T7E1 mismatch cleavage assay reactions for negative (homoduplex) and positive (heteroduplex) control DNA solutions and DNA obtained from the founder of line 34. The presence of the bands indicated by the red arrows confirms that line 34 has a disrupted Prnp allele. (C) Capillary western images confirming that PrP<sup>C</sup> expression is undetectable in brain homogenates from homozygous line 33 mice. Sha31 and 12B2 are two different PrP<sup>C</sup> antibodies. New abbreviation: KO, knockout.

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knockout line (Prnp^{ZH3/ZH3}) was recently generated using a transcription activator-like effector nuclease [29], an alternative gene editing methodology. In line with prior studies, we have designated the FVB/NJ mice bearing this frameshifted allele as Prnp^{Edm/Edm}.

With regards to the goal of functional expression of eCas9 in the germline, we found that use of the Prl3b1 promoter in a transgene construct did not yield expression in the germline of...
male mice in contrast to a previous report [45]. We had searched for male germline-specific promoters due to earlier findings that Cas9 expression in the female germline of Anopheles stephensi mosquitoes may lead to Cas9 persistence in the egg, causing NHEJ-induced indels that render the target gene resistant to the drive mechanism [32]. However, after we had begun our mouse transgenesis experiments, the first demonstration of gene drive in a mammalian species (the mouse) was published by Grunwald et al. (2019) [52]. Interestingly, the authors of this study found that the driving allele was only able to propagate when Cas9 was expressed in the female germline—the opposite result to the mosquito study. Moreover, even when crosses with the male Cas9-expressing mice are excluded from the calculation, the most effective genetic strategy tested achieved a drive efficiency of only 44%, which is likely insufficient for spreading null alleles in a wild population, particularly given that mammals have much longer generation times than insects. The authors speculated that the low efficiency may have been because precise matching of the timing of Cas9 expression to the window in which meiotic recombination occurs is more important in mammals than in insect species. Given these findings and the lack of additional reports of mammalian gene drive since the study by Grunwald et al. (2019) was published, the practicality of this approach in mammals remains unclear. Nevertheless, in the absence of suitably effective control strategies for CWD, we consider it important to explore all possible solutions, including gene drive.

**CRISPR-induced alleles and genetic resistance to prion infection**

Further work on the aforementioned genetic strategies raises the question of the optimal Prnp allele to pursue for in vivo experiments and the fine-structure repertoire of CRISPR-induced mutations. Surprisingly, we found a preponderance of in-frame deletions in our sample set that would affect the N-terminal natively disordered region of PrP\textsuperscript{C} but would spare the globular C-terminal domain. In addition to the 1 bp insertion identified in founder 33, we detected three Prnp alleles with in-frame deletions of varying size when testing the Prnp-directed gRNAs in vivo. Repair of WT Cas9-induced DSBs by classical NHEJ is either error-free or responsible for very small indels, with a bias towards deletions and a median size of 3 bp reported previously [53]. However, two of the mutated alleles we identified contained rather large deletions: 21 bp in one allele and a double deletion of 48 bp and 27 bp in the other. Such deletions are more likely to be caused by an alternative NHEJ pathway called microhomology-mediated end-joining, which can repair DNA when end resection leads to single-strand overhangs with microhomologies of \(\geq 5\) bp [54, 55]; for each of the larger deletions we obtained, perfect microhomologies of 6–8 bp are present immediately 5’ of the deleted sequence and at the 3’ end of the deleted sequence. Nonetheless, the mechanism behind the double deletion is not clear, because the 27 bp deleted sequence starts almost 50 bp away from the predicted Cas9 cleavage site.

Microhomologies occur frequently in the sequence encoding the N-terminal domain of PrP\textsuperscript{C} due to the presence of amino acid repeats in this part of the protein (GGN/SRYP hexarepeats and PHGGG/SWGQ octarepeats), with the codon usage often being similar. Therefore, the microhomologies are usually in frame with each other, which would explain why the large deletions we obtained were, in turn, in frame. This effect is problematic for a gene drive mechanism based on the spread of a null Prnp allele, because DSBs will sometimes be repaired by mechanisms other than HDR even in the case of a highly efficient drive; in such situations, classical NHEJ repair would be ideal, because it is likely to produce frameshift mutations that would eliminate the production of PrP\textsuperscript{C}. In contrast, the large in-frame deletions we have observed would generate permanent resistance to the drive mechanism (due to disruption of the CRISPR protospacer sequence) without greatly affecting the protein product of the gene.
and, by extension, susceptibility to CWD. Certain PrP deletion mutants (e.g., Δ32–121, Δ32–134, Δ94–134) are associated with lethal neurodegenerative phenotypes in mice [56, 57], but these phenotypes require the deletion to extend into the hydrophobic core of the protein (residues ~112–133). None of the deletions generated in our study fit this criteria, although we cannot completely exclude the possibility that sufficiently large deletions could be created. To avoid generating these in-frame deletions, the Prnp gRNA could be targeted to a more 3’ prime position away from the microhomology-dense region, but this would risk generating toxic truncated protein products similar to the pathogenic stop codon allele Y145X [58]. As an alternative to null Prnp alleles entirely, protective amino acid substitutions could be introduced by deploying two gRNAs simultaneously to target sequences 5’ and 3’ of the substitution site in order to produce a modified coding sequence by a double homologous recombination approach [59]–akin to the method used to make knock-in mice. Variant Prnp ORFs to be considered might include the G127V polymorphism (equivalent to G130V in cervid PrP<sup>C</sup>), which appears to be protective against all human prion strains [60]. Avoiding fully penetrant null alleles would also alleviate the potential for loss of PrP<sup>C</sup> expression to cause the peripheral neuropathy phenotype that has been identified in PrP<sup>C</sup>-null mice and goats [28–30]. Gene drive-enabled variant Prnp alleles could then be mobilized in tandem with “gene brake” safety measures [61, 62], which are likely to be necessary for safe use of gene drive in a real world setting and could be important for gaining general public acceptance [63].

In conclusion, although we encountered various technical challenges, including issues with obtaining germline expression of Cas9, that prevented us from developing a complete gene drive mechanism capable of spreading resistance to prion infection, we did succeed in generating reagents able to modify the murine Prnp locus in cultured cells via HDR of Cas9-induced DSBs and created a line of PrP<sup>C</sup>-knockout mice that could be useful to other researchers in the field. Our study has also helped to underline the challenges associated with gene drive in mammalian species, allowing us to pivot towards investigating other potential approaches for controlling CWD and prion disorders in general.

**Materials and methods**

**Selection of suitable spacer sequences for Prnp gRNAs**

Codons 23–50 of murine Prnp were scanned for suitable gRNA spacer sequences using an algorithm developed in the laboratory of Dr. Feng Zhang (no longer available but previously found at [http://crispr.mit.edu](http://crispr.mit.edu)).

**In vitro cleavage of MoPrP.Xho.wt construct**

RecCas9 (Integrated DNA Technologies [IDT], 147821858), CRISPR-Cas9 tracrRNA (IDT, 147821857) and a CRISPR-Cas9 crRNA containing Prnp-specific spacer sequence #1 (IDT; see S1 Fig, panel B, for sequence) were combined at equimolar amounts, heated to 95˚C for 5 min and cooled to room temperature to create RNP complexes. A linearized “half-genomic” Prnp expression construct (MoPrP.Xho.wt) [42] was incubated with Cas9/gRNA RNP complexes (100 nM) for 1 h at 37˚C in a reaction buffer of 20 mM HEPES, pH 6.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.1 mM EDTA. Reactions were stopped by incubation with Proteinase K (Roche, 3115887001) at ~1 mg/mL for 10 min at 56˚C.

**Agarose gel electrophoresis**

DNA samples were separated by agarose gel electrophoresis with the GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific, SM1331) run alongside. Gels were stained with...
SYBR Safe (Invitrogen, S33102) and images obtained using a Fluor Chem E imager (ProteinSimple).

**Molecular cloning**

The eSpCas9(1.1) expression vector was a gift from Dr. Feng Zhang (Addgene plasmid #71814; http://n2t.net/addgene:71814; RRID:Addgene_71814) [41]. Sense and antisense oligonucleotides matching the selected gRNA spacer sequences (S1 Fig, panel B) were synthesized by IDT, annealed and cloned separately into eSpCas9(1.1) using BbsI.

Generation of the donor vector for HDR consisted of several stages; sequences of the relevant primers and DNA fragments are provided in S1 Table. In stage 1, a gBlock fragment (IDT) containing codons 230–254 of murine Prnp was joined to a PCR product derived from pBud.GFP using a Gibson Assembly Cloning Kit (New England Biolabs, E5510S), thereby inserting the Prnp fragment just before the Gfp stop codon and generating the Gfp-GPI transgene. In stage 2, a ~1.6 kb fragment consisting of the Prnp ORF flanked by 5′ (intron 2) and 3’ sequences (3′ untranslated region) was amplified from WT FVB/NJ mouse genomic DNA and cloned into pBluescript SK+ using SalI and HindIII (to make pBluescript SK+.PrnpHA). The primers for this step were designed so that the protospacer and protospacer-adjacent motif for Prnp gRNA–3 were added at both ends of the Prnp fragment, thereby promoting excision of the DNA cassette in the presence of Cas9 and the Prnp gRNA; this approach has been reported to improve HDR efficiency significantly [47]. In the third and final stage, a PCR product consisting of the Gfp-GPI transgene (minus the CMV promoter and start codon but including the 3′ bovine growth hormone polyadenylation signal sequence from the pBud vector) was joined using Gibson Assembly to a PCR product consisting of the entire pBluescript SK+.PrnpHA vector in linear form. Thus, the Gfp-GPI transgene was inserted approximately into the middle of the 1.6 kb Prnp fragment, thereby splitting the CRISPR protospacer sequence and generating ~0.8 kb homology arms on either side to promote integration by HDR.

Generation of the construct used to make Prl3b1-Cas9 transgenic mice also consisted of several stages; sequences of the relevant primers are provided in S1 Table. In stage 1, the bovine growth hormone polyadenylation signal sequence was amplified from pBud.CE4 (Invitrogen) and cloned into pBluescript SK+ using XmaI and SpeI. In stage 2, in order to add an intron upstream of the site the Cas9 cDNA was to be inserted, we used rabbit genomic DNA to amplify a region of the β-globin gene and cloned it into the construct from stage 1 using HindIII and PstI. In stage 3, an insulator sequence was added to reduce potential positional silencing. We used the human β-globin locus control region, amplified from BAC CTD 3055E11, and cloned it into the construct from stage 2 using KpnI and Xhol. The forward primer used for this step contained a NotI site downstream of the KpnI site to aid in purifying the final transgene from the vector backbone. In stage 4, to help with later cloning steps, we added a linker sequence with phosphorylated overlapping oligonucleotides between Xhol and ClaI sites. This step added AscI, NdeI, AatII and Nhel recognition sequences. In stage 5, we added an AgeI site to the vector using phosphorylated overlapping oligonucleotides between PstI and XmaI sites. In stage 6, the Cas9 coding region was excised from eSpCas9(1.1) using AgeI and NotI and cloned into the construct from stage 5 using the same restriction enzymes. In the seventh and final stage, the Prl3b1 promoter region was amplified by nested PCR from mouse BAC RP23-189A16 and cloned into the Xhol and Nhel sites of the construct from stage 6.

All plasmids were purified at the final stage using an EndoFree Plasmid Maxi Kit (Qiagen, 12362). Sequences were checked by diagnostic restriction digests and, subsequently, by Sanger sequencing. The donor vector for HDR was found to contain a 7 bp deletion (of a repetitive tract of G nucleotides) within the 5′ Prnp homology arm. However, this error was >200 bp
upstream of the expected Cas9 cleavage site and so was considered unlikely to affect HDR efficiency to any great extent.

Cell culture and transfections
RK13 cells stably expressing WT murine PrP<sup>C</sup> (clone WT-5) had been generated from the parental RK13 cell line (ATCC, CCL-37) as part of an earlier study [42]. WT-5 RK13 and N2a cells (ATCC, CCL-131) were routinely cultured at 37˚C in 5% CO<sub>2</sub> with 95% humidity in DMEM (Gibco, LS11885084) containing 10% (v/v) fetal bovine serum (FBS; Life Technologies, LS12483020) and 1% (v/v) penicillin-streptomycin solution (pen-strep; Gibco, LS15140122). Transient transfections were performed using Lipofectamine 2000 (Invitrogen, 116608027) or Lipofectamine 3000 (Invitrogen, L3000008) 24 h after cells were seeded into plates in DMEM supplemented with 10% (v/v) FBS.

Fluorescence-activated cell sorting and DNA isolation
Cells were detached using Accutase (Corning, 25058C) and transferred to collection tubes (Corning, 352058). Working on ice, cells were washed, blocked (10 min), incubated with Zombie Aqua dye (Biolegend, 423101; diluted 1:250 from the reconstituted DMSO stock), incubated with SAF83 anti-PrP antibody (Cayman Chemicals, 189765; diluted 1:50; 20 min), washed again, incubated with goat anti-mouse Dylight 649 secondary antibody (Thermo Scientific, 35515; diluted 1:100; 20 min), and washed once more. PBS (Boston Bioproducts, BM-220) supplemented with 25 mM HEPES, 1% (v/v) FBS, 0.5 mM EDTA, and 1% (v/v) pen-strep was used as the buffer for all the above steps (except for adjustment of FBS to 10% for the blocking step). Solutions were exchanged by centrifugation followed by resuspension of the pellet in the new buffer. FACS based on PrP<sup>C</sup> signal intensity was performed by staff at the University of Alberta’s Flow Cytometry Facility using a BD FACSAria III instrument, with FACSDiva Version 6.1.3 (BD Biosciences) used for analysis. The intense fluorescence of GFP produced by the stably integrated expression vector interfered with Zombie Aqua-based live/dead cell discrimination. Instead, tight gating based on side scatter (SSC) area vs forward scatter (FSC) area plots was used to exclude dead cells. Additional gates based on SSC and FSC width vs height plots were applied to exclude cell clumps. A final gate was applied to select the ~10% of cells with the lowest PrP<sup>C</sup> signals, which were centrifuged, resuspended in PBS containing 25 mM HEPES, and centrifuged again. DNA was isolated from cell pellets using a DNAeasy Blood and Tissue Kit (Qiagen, 69504).

T7E1 mismatch cleavage assays
Prnp<sup>C</sup> fragments were amplified using AccuPrime Taq DNA Polymerase, High Fidelity (Invitrogen, LS12346086; see S1 Table for primer sequences). After agarose gel electrophoresis, DNA concentrations were estimated by comparison to the GeneRuler 1 kb Plus DNA Ladder; band intensities were quantified using ImageJ. Hybridization and T7E1 incubation steps were performed according to the Alt-R Genome Editing Detection Kit instructions (Integrated DNA Technologies, 1075931), except that incubation with T7E1 was shortened to 30 min. Reactions were stopped by addition of EDTA to ~25 mM.

Junction PCRs
PCRs were performed using AccuPrime Taq DNA Polymerase, High Fidelity (Invitrogen, LS12346086) and primers (see S1 Table) that would only generate an amplicon if the Gfp-GPI transgene insert was present. PCR products were extracted from agarose gels using a MinElute
Gel Purification Kit (Qiagen, 28604), ligated into the pCR2.1–TOPO TA vector using a cloning kit (Invitrogen, 450641) and transformed into One Shot Max Efficiency DH5α-T1R Competent Cells (Invitrogen, 12297016). Plasmid DNA samples were subjected to diagnostic restriction digests followed by Sanger sequencing.

**Generation of transgenic mice**

Animal handling procedures and husbandry were in accordance with Canadian Council on Animal Care guidelines and approved by the University of Alberta institutional ethics review (AUP00000356, AUP00000358). To test Prnp gRNAs in vivo, RNP complexes were prepared as described previously for the in vitro cleavage experiment, except that the crRNA contained Prnp-specific spacer sequence #3 (see S1 Fig, panel B, for sequence). RNP complexes were electroporated into fertilized FVB/NJ mouse oocytes at the University of Alberta’s Transgenic Core Facility. Zygotes were washed into 10 μL Opti-MEM (Gibco, 31985062) and mixed with the RNP complexes in 10 μL Opti-MEM. The mixture was transferred to a 0.1 cm electroporation cuvette (Bio-Rad). Electroporation was performed in a Bio-Rad GenePulser XCell using three pulses of 3.0 ms at 30V with a 100 ms interval between pulses. Zygotes were cultured overnight in KSO media (Millipore, MR-121-D) in a MINC benchtop incubator (Cook Medical; 37˚ C, 5% CO₂, and 5% O₂/nitrogen). Viable embryos were surgically transferred to pseudopregnant CD1 female mice on the following day, except for when Prnp was sequenced in blastocyst-stage embryos; in these cases, embryos were cultured in vitro to 5.5 dpc before being washed with M2 medium (Sigma-Aldrich, M7167) and transferred individually to PCR tubes. Embryos were digested for 30 min at 56˚ C in 10 μl of PBDN buffer (“PCR Buffer Nonionic Detergents” [64]), consisting of 50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% (v/v) NP-40 substitute, 0.45% Tween-20, and 0.1 mg/ml proteinase K (Invitrogen, 25530049). After incubating at 95˚C for 10 min to inactivate proteinase K, amplification of Prnp by PCR was performed using AccuPrime Taq DNA Polymerase, High Fidelity (Invitrogen, LS12346086; primers listed in S1 Table).

Prl3b1-Cas9 transgenic mice were generated by microinjection of the purified construct into the pronuclei of 0.5 dpc FVB/NJ fertilized oocytes using a XenoWorks digital microinjector (Sutter). Viable zygotes were surgically transferred to pseudopregnant CD1 female mice.

**Isolation and purification of DNA from mice**

Ear or tail tissue samples were digested overnight at 55˚C with 0.8 mg/mL proteinase K (Invitrogen, 25530049) in a buffer of 50 mM Tris, pH 8.0, 100 mM NaCl and 1% (w/v) SDS. An equal volume of Buffer-Saturated Phenol was added (Invitrogen, 15513047) and samples were centrifuged at 16000 × g. The DNA was precipitated using 95% (v/v) ethanol, centrifuged, washed with 70% ethanol and resuspended in TE buffer.

**Sanger sequencing**

Sanger sequencing of DNA samples was performed by staff at the Molecular Biology Services Unit of the Department of Biological Sciences, University of Alberta (see S1 Table for details of sequencing primers). DNA chromatograms were checked using SnapGene Viewer version 6.0.2 and sequence alignments were performed using Serial Cloner version 2.6.1.

**Homogenization of tissues and capillary western assays**

Adult mice were euthanized by cervical dislocation and tissues were immediately extracted and frozen on dry ice. Embryos together with their respective placentas were extracted from
pregnant females at 15 dpc. Mouse brains were homogenized as described previously [65]. Other tissue types were homogenized in a buffer of 50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40 substitute, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, and a protease inhibitor cocktail (Roche, 04693159001) using needles of decreasing diameter (urogenital tract tissues) or a Dounce homogenizer (embryo–placenta tissues). Homogenates of all tissues other than brain were clarified by centrifugation and total protein concentrations in the supernatants were determined by bicinchoninic acid assay (Thermo Scientific, 0023225). Capillary western plates were prepared, run using a Wes instrument (ProteinSimple) and analysed using the associated Compass software as described previously [65]. Primary antibodies were Sha31 anti-PrP (Spi-Bio Inc., A03213; diluted 1:10000), 12B2 anti-PrP (see Ref. [51]; diluted 1:500) and anti-beta-tubulin (Novus Biologicals, NB600-936; diluted 1:200). On occasions when the Total Protein Detection Module (ProteinSimple, DM-TP01) was used for some capillaries, plates were prepared according to the module instructions and were run using the default Total Protein protocol in the Compass software.

Supporting information

S1 Fig. Further information on selected Prnp guide RNAs. (A) The top 10 gRNA spacers within codons 23–50 of murine Prnp that were identified by the gRNA design algorithm previously found at http://crispr.mit.edu. The scores reported by the algorithm derive from a combination of the on-target efficiency rating and the number of potential off-target sites. (B) The chosen Prnp gRNA spacer sequences are shown aligned to the murine Prnp ORF (NCBI Accession #: NM_011170.3). Due to the preference of the U6 promoter for an initiating G nucleotide, a deliberately mismatched initial G nucleotide was included for gRNA–2 and gRNA–3. (C) The highest-scoring predicted cleavages for the second spacer listed in panel A (contained within gRNA–3). Data derives from the same gRNA design algorithm. Sanger sequencing of a PCR amplicon containing the potential off-target site was attempted for each of the six shown. Red highlighting indicates that suitable primers could be designed and that readable sequencing data was obtained.

(TIF)

S2 Fig. FACS of RK13 cells transiently expressing Cas9 and Prnp gRNAs. WT-5 RK13 cells underwent FACS 48 h after transfection with eSpCas9(1.1) expression plasmids containing Prnp gRNA–1, –2 or –3, or no gRNA. Example data for gRNA–1 is shown here, but the same process was applied to each sample. Tight gating based on the side scatter area (SSC-A) vs forward scatter area (FSC-A) plot (A) was used to exclude dead cells. (B, C) Gates were applied based on SSC and FSC width (W) vs height (H) plots to exclude cell clumps. (D) A gate was applied to select the ~10% of cells with the lowest PrP C signals (P4). (E) Summary table showing the cell counts in each population.

(TIF)

S3 Fig. Checking junctions for correct insertion of the reporter transgene into the N2a cell genome. (A) Diagnostic digests of pCR2.1–TOPO containing junction PCR products (purified from the gel shown in Fig 3C) were prepared using the indicated restriction enzymes. The expected band sizes were 4.0 and 1.3 kb or 5.1 and 0.25 kb for the 3′ junction PCR products (depending on the orientation in which the PCR product inserted into pCR2.1–TOPO) and 3.9 and 1.2 kb for the 5′ junction products (irrespective of orientation in pCR2.1–TOPO). All of the 5′ junction samples and 5/6 of the 3′ junction samples produced the expected band patterns. (B, C) Example Sanger sequencing chromatograms for minipreps #1 and #7 showing that the sequences obtained matched the expected junction PCR products, indicating the
absence of unwanted indels around the junction sites. For the expected sequences, underlined letters correspond to the Gfp-GPI transgene sequence and non-underlined capitals to the Prnp homology arms.

S4 Fig. Sequencing data from a mouse embryo with CRISPR/Cas9-induced deletions within Prnp. (A) Sanger sequencing chromatogram for embryo 20 derived from fertilized FVB/NJ oocytes that had been electroporated with recCas9/Prnp gRNA–3 RNP complexes. The numbers below the chromatogram indicate the position in the sequencing read rather than the Prnp ORF. The data suggested that two relatively large deletions were present within one Prnp allele. (B) To eliminate the mixed sequence, the original sequencing PCR product was ligated into pCR2.1–TOPO and fresh Sanger sequencing data were obtained from a clone suggested to contain the deletion(s) by diagnostic restriction digests. The alignment with the WT Prnp ORF sequence confirms that two deletions of 48 and 27 bp were present in one Prnp allele of embryo 20. The exact start and end sites of the deletions cannot be determined due to the presence of microhomologies (underlined). (C) The predicted changes to the N-terminal amino acid sequence of mature PrP in embryo 20 based on the deletions observed within the Prnp ORF.

S5 Fig. Uncropped agarose gel and capillary western images. The figure panels that the images correspond to are indicated. A red “X” is used to indicate that a lane was omitted from the final figure panel.

S1 Table. Primer and DNA sequences. The ends of the gBlock fragment (underlined) were complementary to the pBud.GFP forward (For) and reverse (Rev) primers to facilitate Gibson Assembly; the sequence in lower case corresponds to codons 230–254 of murine Prnp. The PrnpHA–For and–Rev primers both start with 6 random nucleotides followed by a SalI site (For) or HindIII site (Rev), the protospacer sequence plus the protospacer-adjacent motif for Prnp gRNA–3 (see S1 Fig, panel B, for sequence), and, finally, the Prnp-specific sequences. To facilitate Gibson Assembly, the GFP–GPI–For1 and–Rev1 primers contained 18 nt 5’ sequences complementary to the ends of the vector fragment amplified by the pB–HA–For and–Rev primers. For the junction PCRs, the Prnp–Intron2–For and GFP–GPI–Rev2 primers were used to analyse the 5’ junction, GFP–GPI–For2 and Prnp–3UTR–Rev the 3’ junction. The M13–For(-20) and M13–Rev primers were provided as part of a TOPO TA cloning kit (Invitrogen, 450641) and were used to sequence the junction PCR products in both directions.

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References

1. Rivera NA, Brandt AL, Novakofski JE, Mateus-Pinilla NE. Chronic Wasting Disease In Cervids: Prevalence, Impact And Management Strategies. Veterinary Medicine-Research and Reports. 2019; 10:123–39. https://doi.org/10.2147/VMRR.S197404 WOS:000488624800001. PMID: 31632898

2. Edmunds DR, Kaufmann MJ, Schumaker BA, Lindzey FG, Cook WE, Kreeger TJ, et al. Chronic Wasting Disease Drives Population Decline of White-Tailed Deer. Plos One. 2016; 11(8). https://doi.org/10.1371/journal.pone.0161127 WOS:000382877200017. PMID: 27575545

3. Igel-Egalon A, Beringue V, Rezaei H, Sibille P. Prion Strains and Transmission Barrier Phenomena. Pathogens. 2018; 7(1). https://doi.org/10.3390/pathogens7010005 WOS:000422657000005. PMID: 29301257

4. Houston F, Andreoletti O. Animal prion diseases: the risks to human health. Brain Pathology. 2019; 29(2):248–62. https://doi.org/10.1111/bpa.12696 WOS:000459921800010. PMID: 30588682

5. Herbst A, Velasquez CD, Triscott E, Aiken JM, McKenzie D. Chronic Wasting Disease Prion Strain Emergence and Host Range Expansion. Emerging Infectious Diseases. 2017; 23(9):1598–600. https://doi.org/10.3201/eid2309.161474 WOS:000407786000031. PMID: 28820384

6. Race B, Williams K, Chesebro B. Transmission studies of chronic wasting disease to transgenic mice overexpressing human prion protein using the RT-QuIC assay. Veterinary Research. 2019;50. https://doi.org/10.1186/s13567-019-0626-2 WOS:000456630200001. PMID: 30670087

7. Mysterud A, Edmunds DR. A review of chronic wasting disease in North America with implications for Europe. European Journal of Wildlife Research. 2019;65(2). https://doi.org/10.1007/s10344-019-1260-z WOS:000459400300002.

8. Goni F, Mathiason CK, Yim L, Wong KL, Hayes-Klug J, Nalls A, et al. Mucosal immunization with an attenuated Salmonella vaccine partially protects white-tailed deer from chronic wasting disease. Vaccine. 2015; 33(5):726–33. https://doi.org/10.1016/j.vaccine.2014.11.035 WOS:000349503700022. PMID: 25539804

9. Taschuk R, Scruten E, Woodbury M, Cashman N, Potter A, Griebel P, et al. Induction of PrPSc-specific systemic and mucosal immune responses in white-tailed deer with an oral vaccine for chronic wasting disease. Prion. 2017; 11(5):368–80. https://doi.org/10.1080/19336896.2017.1367083 WOS:000416057100008. PMID: 28968152

10. Abdelaziz DH, Thapa S, Brandon J, Maybee J, Vankuppeveld L, McCormell R, et al. Recombinant prion protein vaccine vaccination of transgenic elk PrP mice and reindeer overcomes self-tolerance and protects mice against chronic wasting disease. Journal of Biological Chemistry. 2018; 293(51):19812–22. https://doi.org/10.1074/jbc.RA118.004810 WOS:000454294900025. PMID: 30397182

11. Wood ME, Griebel P, Huizenga ML, Lockwood S, Hansen C, Potter A, et al. Accelerated onset of chronic wasting disease in elk (Cervus canadensis) vaccinated with a PrPSc-specific vaccine and housed in a prion contaminated environment. Vaccine. 2018; 36(50):7737–43. https://doi.org/10.1016/j.vaccine.2018.10.057 WOS:000454463800019. PMID: 30417779

12. Vey M, Pilkuhn S, Wille H, Nixon R, Dearmond SJ, Smart EJ, et al. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proceedings of the National Academy of Sciences of the United States of America. 1996; 93(25):14945–9. https://doi.org/10.1073/pnas.93.25.14945 WOS:A1996VY44000136. PMID: 8962161

13. Castle AR, Gill AC. Physiological Functions of the Cellular Prion Protein. Front Mol Biosci. 2017; 4:19. Epub 2017/04/22. https://doi.org/10.3389/fmolb.2017.00019 PMID: 28428956; PubMed Central PMCID: PMC5382174.
14. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science. 1982; 216(4542):136–44. https://doi.org/10.1126/science.6801762 WOS:A1982NH46800006. PMID: 6801762

15. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell. 1983; 35(1):57–62. https://doi.org/10.1016/0092-8674(83)90207-6 WOS:A1983RR00100011. PMID: 6414721

16. Prusiner S, Oesch B, Walchli M, Westaway D, McKinley M, Teplow D, et al. Molecular-cloning studies demonstrate that cellular genomic DNA encodes the scrapie prion protein. Clinical Research. 1985; 33(2):A565–A. WOS:A1985AEY9302427.

17. Mays CE, Kim C, Haldiman T, van der Merwe J, Lau A, Yang J, et al. Prion disease tempo determined by host-dependent substrate reduction. Journal of Clinical Investigation. 2014; 124(2):40–8. https://doi.org/10.1172/JCI72241 WOS:000331413300014. PMID: 24430187

18. Raymond GJ, Zhao HT, Race B, Raymond LD, Williams K, Swayze EE, et al. Antisense oligonucleotides extend survival of prion-infected mice. Jci Insight. 2019; 4(16). https://doi.org/10.1172/jci.insight.131175 WOS:000482229000024. PMID: 31361599

19. Bueler H, Fischer M, Lang Y, Bluetmann H, Lipp HP, Dearmond SJ, et al. Normal development of mice lacking the neuronal cell-surface PrP protein. Nature. 1992; 356(6370):577–82. https://doi.org/10.1038/356577a0 WOS:A1992HP03100039. PMID: 1373228

20. Bue1er H, Fischer M, Lang Y, Bluetmann H, Lipp HP, Dearmond SJ, et al. Normal development of mice lacking the neuronal cell-surface PrP protein. Nature. 1992; 356(6370):577–82. https://doi.org/10.1038/356577a0 WOS:A1992HP03100039. PMID: 1373228

21. Prusiner SB, Groth D, Serban A, Koehler R, Foster D, Torchia M, et al. Ablation of the prion protein (prp) gene in mice prevents scrapie and facilitates production of anti-prp antibodies. Proceedings of the National Academy of Sciences of the United States of America. 1993; 90(22):10608–12. https://doi.org/10.1073/pnas.90.22.10608 WOS:A1993MH32200041. PMID: 7902565

22. Bue1er H, Fischer M, Lang Y, Bluetmann H, Lipp HP, Dearmond SJ, et al. Normal development of mice lacking the neuronal cell-surface PrP protein. Nature. 1992; 356(6370):577–82. https://doi.org/10.1038/356577a0 WOS:A1992HP03100039. PMID: 1373228

23. Manson JC, Clarke AR, Hooper ML, Aitchison L, McConnell I, Hope J. 129/ola mice carrying a null mutation in prp that abolishes messenger-RNA production are developmentally normal. Molecular Neurobiology. 1994; 8(2–3):121–7. https://doi.org/10.1007/BF02780662 WOS:A1994PD12000007. PMID: 7999308

24. Zhu C, Li B, Yu G, Chen J, Yu H, Chen J, et al. Production of Prnp(-/-) goats by gene targeting in adult fibroblasts. Transgenic Research. 2009; 18(2):163–71. https://doi.org/10.1007/s11248-008-9220-5 WOS:000264108100002. PMID: 16821027

25. Yu GH, Chen JQ, Xu YY, Zhu CH, Yu HQ, Liu SG, et al. Generation of Goats Lacking Prion Protein. Molecular Reproduction and Development. 2009; 76(1):3–9. https://doi.org/10.1002/mrd.20960 WOS:000264164700001. PMID: 18951376

26. Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, et al. Production of cattle lacking prion protein. Nature Biotechnology. 2007; 25(1):132–8. https://doi.org/10.1038/nbt.1271 WOS:000243439100003. PMID: 17195841

27. Benestad SL, Austbo L, Tranulis MA, Espenes A, Olsaker I. Healthy goats naturally devoid of prion protein. Veterinary Research. 2012; 43. https://doi.org/10.1186/1297-9716-43-87 WOS:000314020600001. PMID: 23249298

28. Bremer J, Baumann F, Tiberi C, Wessig C, Fischer H, Schwarz P, et al. Axonal prion protein is required for peripheral myelin maintenance. Nature Neuroscience. 2010; 13(3):310–9. https://doi.org/10.1038/nn.2485 WOS:000274860100012. PMID: 20998419

29. Nuvolone M, Hermann M, Sorce S, Russo G, Tiberi C, Schwarz P, et al. Strictly co-isogenic C57BL/6J-Prnp(-/-) mice: A rigorous resource for prion science. Journal of Experimental Medicine. 2016; 213(3):313–27. https://doi.org/10.1084/jem.20151610 WOS:000373390300003. PMID: 26926995

30. Skedsmo FS, Malachin G, Vage DI, Hammerovd MM, Salvesen O, Ersdal C, et al. Demyelinating polyneuropathy in goats lacking prion protein. Faseb Journal. 2020; 34(2):2359–75. https://doi.org/10.1096/fj.201902588R WOS:000514663600030. PMID: 31907995

31. Burt A, Crisanti A. Gene Drive: Evolved and Synthetic. Acs Chemical Biology. 2018; 13(2):343–6. https://doi.org/10.1021/acschembio.7b01031 WOS:000426012800008. PMID: 29400944

32. Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E, et al. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112(49): E6736–E43. https://doi.org/10.1073/pnas.1521077112 WOS:000365989800007. PMID: 26598698
A gene drive approach to prion disease resistance

33. Hammond A, Galizi R, Kyrou K, Simon A, Siniscalchi C, Katsanos D, et al. A CRISPR-Cas9 gene drive system-targeting female reproduction in the malaria mosquito vector Anopheles gambiae. Nature Biotechnology. 2016;34(1):78–83. https://doi.org/10.1038/nbt.3439 WOS:000368758200030. PMID: 26641531

34. Kyrou K, Hammond AM, Galizi R, Kranjc N, Burt A, Beaghton AK, et al. A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nature Biotechnology. 2018;36(11):1062–9. https://doi.org/10.1038/nbt.4245 WOS:000450374000019. PMID: 30247490

35. Adolfi A, Gantz VM, Jasinskiene N, Lee HF, Hwang K, Terradas G, et al. Efficient population modification gene-drive rescue system in the malaria mosquito Anopheles stephensi. Nature Communications. 2020;11(1). https://doi.org/10.1038/s41467-020-19426-0 WOS:000518433000006. PMID: 33144570

36. Dearden PK, Gemmell NJ, Mercier OR, Lester PJ, Scott MJ, Newcomb RD, et al. The potential for the use of gene drives for pest control in New Zealand: a perspective. Journal of the Royal Society of New Zealand. 2018;48(4):225–44. https://doi.org/10.1080/03036758.2017.1385030 WOS:000439895700001.

37. Friedland AE, Tzur YB, Esvelt KM, Colaiacovo MP, Church GM, Calarco JA. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nature Methods. 2013;10(8):741–8. https://doi.org/10.1038/nmeth.2532 WOS:000322453600021. PMID: 23817069

38. Sternburg EL, Dias KC, Karginov FV. Selection-dependent and Independent Generation of CRISPR/Cas9-mediated Gene Knockouts in Mammalian Cells. Jove-Journal of Visualized Experiments. 2017;2017(124). https://doi.org/10.3791/55903 WOS:000415711000119. PMID: 28654078

39. Moore RC, Lee IY, Silverman GL, Harrison PM, Strome R, Heinrich C, et al. Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein Doppel. Journal of Molecular Biology. 1999;292(4):787–817. https://doi.org/10.1006/jmbi.1999.3108 WOS:000083134500005. PMID: 10525406

40. Mehrabian M, Brethour D, Maclsaac S, Kim JK, Gunawardana CG, Wang H, et al. CRISPR-Cas9-Based Knockout of the Prion Protein and Its Effect on the Proteome. Plos One. 2014;9(12). https://doi.org/10.1371/journal.pone.0114594 WOS:000347515300006. PMID: 25490046

41. Slaymaker IM, Gao LY, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. Science. 2016;351(6268):84–8. https://doi.org/10.1126/science.aad5227 WOS:000367364200048. PMID: 26628643

42. Lau A, McDonald A, Daude N, Mays CE, Walter ED, Aglietti R, et al. Octarepeat region flexibility impacts prion function, endoproteolysis and disease manifestation. Embo Molecular Medicine. 2015;7(3):339–56. WOS:000350767600009. https://doi.org/10.15252/emmm.201404588 PMID: 25661904

43. Raymond GJ, Olsen EA, Lee KS, Raymond LD, Bryant PK, Baron GS, et al. Inhibition of protease-resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease. Journal of Virology. 2006;80(2):596–604. https://doi.org/10.1128/JVI.80.2.596-604.2006 WOS:000234382900006. PMID: 16378962

44. Paladino S, Lebretton S, Tivodar S, Campana V, Tempre R, Zurrzolo C. Different GPI-attachment signals affect the oligomerisation of GPI-anchored proteins and their apical sorting. Journal of Cell Science. 2008;121(24):4001–7. https://doi.org/10.1242/jcs.036038 WOS:000261378700002. PMID: 19056670

45. Al-Soudy AS, Nakanishi T, Mizuno S, Hasegawa Y, Shawki HH, Katoh MC, et al. Germline recombination in a novel Cre transgenic line, Prl3b1-Cre mouse. Genesis. 2016;54(7):389–97. https://doi.org/10.1002/dvg.22944 WOS:000380040300003. PMID: 27124574

46. Simmons DG, Rawn S, Davies A, Hughes M, Cross JC. Spatial and temporal expression of the 23 murine Prolactin/Placental Lactogen-related genes is not associated with their position in the locus. Bmc Genomics. 2008;9 https://doi.org/10.1186/1471-2164-9-352 WOS:000259136800021. PMID: 18696008

47. Zhang JP, Li XL, Li GH, Chen WQ, Arakaki C, Botimer GD, et al. Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. Genome Biology. 2017;18(1). https://doi.org/10.1186/s13059-017-1164-8 WOS:000394828100003. PMID: 28654078

48. Mays CE, Yeom J, Kang HE, Bian JF, Khaychuk V, Kim Y, et al. In Vitro Amplification of Misfolded Prion Protein Using Lysate of Cultured Cells. Plos One. 2011;6(3). https://doi.org/10.1371/journal.pone.0018047 WOS:000289053800015. PMID: 21464935

49. Chasseigneaux S, Pastore M, Britton-Davidian J, Manie E, Stern MH, Callebert J, et al. Genetic heterogeneity versus molecular analysis of prion susceptibility in neuroblasm a N2a sublines. Archives of Virology. 2008;153(9):1693–702. https://doi.org/10.1007/s00705-008-0177-8 WOS:000258717500008. PMID: 18696008

50. Feraudet C, Morel N, Simon S, Volland H, Frobert Y, Creminon C, et al. Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. Journal of
51. Langeveld JPM, Jacobs JG, Erkens JHF, Bossers A, van Zijderve ld FG, van Keulen LJM. Rapid and
discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. BMC veterinary
research. 2006; 2:19. https://doi.org/10.1186/1746-6148-2-19 MEDLINE:PMID: 16674717.

52. Grunwald HA, Gantz VM, Poplawski G, Xu XRS, Bier E, Cooper KL. Super-Mendelian inheritance medi-
ated by CRISPR-Cas9 in the female mouse germline. Nature. 2019; 566(7742): 105–+. https://doi.or g/
10.1038/s41586-019-0875-2 WOS:000457981800047. PMID: 30675057

53. Bothmer A, Phadke T, Barrera LA, Margulies CM, Lee CS, Buquicchio F, et al. Characterization of the
interplay between DNA repair and CRISPR/Cas9-induced DNA lesions at an endogenous locus. Nature
Communications. 2017;8. https://doi.org/10.1038/ncomms13905 WOS:000391366400001.

54. Ceccaldi R, Rondinelli B, D’Andrea AD. Repair Pathway Choices and Consequences at the Double-
Strand Break. Trends in Cell Biology . 2016; 26(1):52–6 4. https://doi. org/10.1016/j .tcb.2015.07 .009
WOS:000368206000007. PMID: 26437586

55. Shmerling D, Hegyi I, Fischer M, Blattler T, Brandner S, Gotz J, et al. Expression of amino-terminally
truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. Cell. 1998; 93(2):203–14.
https://doi.org/10.1016/s0092-8674(00)81572- x WOS:000073174000007. PMID: 9567813

56. Acosta S, Fiore L, Carota IA, Oliver G. Use of two gRNAs for CRISPR/Cas9 improves bi-allelic homolo-
gous recombin ation efficiency in mouse embryonic stem cells. Genesis. 2018; 56(5). https:// doi.org/10.
1002/dvg.23212 WOS:000341107000003. PMID: 29676032

57. Scavizzi F, Ryder E, Newman S, Raspa M, Gleeson D, Wardle-Jones H, et al. Blastocyst genotyping
for quality control of mouse mutant archives: an ethical and econom ical approach. Transgen ic
Research. 2015; 24(5):921– 7. https://doi.org/10.1007/s11248 -015-9897-1 WOS:0003614592000013.
PMID: 26178246

58. Castle AR, Daude N, Gilch S, Westaway D. Application of high-throughput, capillary-based Western
analysis to modulated cleavage of the cellular prion protein. Journal of Biological Chemistry. 2019; 294
(8):2642–50. https://doi.org/10.1074/jbc.RA118.006367 WOS:000459601600007. PMID: 30578300