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CRISPR/Cas9 does not facilitate stable expression of long C9orf72 dipeptides in mice

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ABSTRACT

A C9orf72 repeat expansion is the most common cause of both frontotemporal dementia and motor neuron disease. The expansion is translated to produce dipeptide repeat proteins (DPRs), which are toxic in vivo and in vitro. However, the mechanisms underlying DPR toxicity remain unclear. Mouse models which express DPRs at repeat lengths found in human disease are urgently required to investigate this. We aimed to generate transgenic mice expressing DPRs at repeat lengths of >1000 using alternative codon sequences, to reduce the repetitive nature of the insert. We found that although these inserts did integrate into the mouse genome, the alternative codon sequences did not protect from instability between generations. Our findings suggest that stable integration of long DPR sequences may not be possible. Administration of viral vectors after birth may be a more effective delivery method for long repeats.

1. Introduction

A large G_4C_2 repeat expansion in C9orf72 is the most common genetic cause of both frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), accounting for ~1 in 12 cases (DeJesus-Hernandez et al., 2011; Renton et al., 2011). There are 3 potential mechanisms through which this expansion could cause neurodegeneration: (1) haploinsufficiency (C9orf72 mRNA expression is reduced in patients; Shi et al., 2018; Waite et al., 2014), (2) production of toxic RNA foci that sequester important proteins (DeJesus-Hernandez et al., 2011), or (3) repeat-associated non-ATG translation (RAN-translation) of the expansion to produce 5 dipeptide repeat proteins (DPRs) that aggregate in patient neurons (Ash et al., 2013; Mori et al., 2013). These dipeptides are highly toxic in cell culture, *Drosophila* and zebrafish models (Bennion Callister et al., 2016; Kwon et al., 2014; Mizielinska et al., 2014; Swaminathan et al., 2018; Wen et al., 2014; Zhang et al., 2014), suggesting that DPRs contribute to disease pathogenesis in C9orf72-linked FTD/ALS (C9FTD/ALS). However, the mechanisms underlying DPR toxicity remain unclear, and in vivo mammalian models of DPR pathology are urgently required to address this.

A number of mouse models have recently been generated which express DPRs using alternative codon sequences to produce the same amino acid sequence found in disease, in the absence of the repetitive G_4C_2 RNA. Two different mouse models expressing GR both exhibited neuronal loss, microgliosis, and astrogliosis (Choi et al., 2019; Zhang et al., 2018); however, the behavioral phenotypes reported varied between these 2 studies. Expression of GR80 caused increased anxiety-like behavior and impaired social interaction (Choi et al., 2019), whereas mice expressing GR100 exhibited impairments in motor function and memory, but no anxiety-like behavior was observed in an open-field test and social interaction tests were not reported to be performed (Zhang et al., 2018). Similar variation was reported in 2 studies of GA expression in mice, with impaired motor function, memory deficits, and increased anxiety observed in a GA50 (Zhang et al., 2016) mouse but spatial memory and muscle function spared in a GA149 mouse (Schludi et al., 2017). The GA149 mouse did however exhibit anxiety-like behavior, abnormal gait and balance, and impaired startle reflexes. The variation between these 2 GA mice may have been caused by methodological differences, since the GA50 construct was expressed throughout the cortex and GA149 was primarily expressed in spinal cord. In both studies, neuronal loss and gliosis were observed in regions containing GA pathology. Finally, 2 mouse lines expressing PR50 and PR28 were recently described. Approximately 60% of mice expressing PR50 died by 4 weeks of age, and neuronal loss, gliosis, and deficits in motor function and memory were observed in those that survived to 3 months (Zhang et al.,

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2019). PR28 mice exhibited motor deficits and anxiety-like behavior as well as reduced survival and decreased body weight, and pathological analysis revealed motor neuron loss and gliosis (Hao et al., 2019).

Taken together, these studies strongly support a key role for DPRs in driving neurodegeneration in C9FTD/ALS, and demonstrate differential effects of each peptide. However, a major limitation of these models is that all express DPRs at considerably shorter repeat lengths than are typically found in patients (usually >1000 repeats) (DeJesus-Hernandez et al., 2011; Mackenzie et al., 2015; Renton et al., 2011). We have previously demonstrated that DPR toxicity closely correlates with repeat length in human cell culture and zebrafish (Bennion Callister et al., 2016; Swaminathan et al., 2018), highlighting the importance of expressing DPRs at disease-relevant lengths when modeling C9FTD/ALS. This was particularly important for AP, which has previously been reported as non-toxic by multiple groups but which was toxic only when expressed at over 1000 repeats in our models (Bennion Callister et al., 2016). No mouse models of AP have been published to date.

Several mouse lines expressing pure G_4C_2 repeats have also been generated, meaning that G_4C_2 repeat RNA is produced along with all 5 DPRs in these mice. Indeed, the first mouse model of C9FTD/ALS to be published was generated by adeno-associated viral administration of $(G_4C_2)66$ after birth, resulting in production of intraneuronal RNA foci and DPR inclusions (Chew et al., 2015). These mice exhibited neuronal loss, gliosis, and TDP-43 pathology, as well as impairments in motor function, social interaction, and increased anxiety-like behavior. Since then, several groups have used bacterial artificial chromosomes (BACs) to express longer G_4C_2 repeat sequences which are more relevant to human disease; however, the pathological and behavioral consequences of G_4C_2 expression are extremely inconsistent between BAC lines (Jiang et al., 2016; Liu et al., 2016; O'Rourke et al., 2015; Peters et al., 2015). No neuronal loss, gliosis, or behavioral phenotypes were observed in BAC mice expressing ~500 repeats (Peters et al., 2015) or in a less stable mouse line expressing a range of repeat lengths between 100 and 1000 (O'Rourke et al., 2015). However, no p62 inclusions were present in either of these lines, implying that DPR translation may only have occurred at very low levels, since GA inclusions are known to co-localize with p62 in other models and in human brain. This would suggest that DPRs production is required for neurodegeneration caused by G_4C_2 repeats, which is supported by previous work in *Drosophila* (Mizielinska et al., 2014).

In contradiction to these initial BAC mice, a more recent model expressing ~450 pure G_4C_2 repeats observed slight neuronal loss in the hippocampus and memory and anxiety phenotypes by 12 months of age, although there were no impairments in motor function or social interaction in these mice, and neuronal loss was not observed in other brain regions (Jiang et al., 2016). Both sense and antisense RNA foci were detected throughout the CNS, although only sense DPRs (GA, GR, and GP) were detected and formed inclusions, perhaps implying that again DPR expression was low overall and that this resulted in a relatively mild neurodegenerative phenotype. A final study using BAC G_4C_2 mice reported considerable phenotypic variation even within cohorts of the same 500 repeat mouse line, with a proportion of animals displaying an "acute disease" phenotype and significantly reduced survival, and the remaining animals developing a less severe, more slowly progressing disease phenotype (Liu et al., 2016). Motor defects, hyperactivity, and increased anxiety-like behaviors were all reported in this line. Overall, these studies demonstrate the technical difficulties in generating a mouse model which stably expresses a pure G_4C_2 repeat sequence at a repeat length relevant to human disease, and which consistently models behavioral phenotypes associated with FTD/ALS. Furthermore, the literature to date strongly supports DPRs as key drivers of neurodegeneration in CFTD/ALS, highlighting the importance of developing mouse models which can be reliably used to investigate disease mechanisms. A limitation of BAC mice which express pure G_4C_2 repeat sequences is that RNA foci and all 5 DPRs may be produced in brain, and therefore it is not possible to distinguish between disease mechanisms caused by each of these species. The alternative codon DPR-only models are more suitable for investigation of the mechanisms through which DPRs trigger neurodegeneration; however, those published to date are limited by expression of short repeat lengths which are not found in patients. We therefore aimed to generate 4 novel transgenic mouse lines using alternative codon sequences to produce GA, GR, PR, or AP at over 1000 repeat units in length, in the absence of G_4C_2 repeat RNA.

The 4 DPR sequences, under the control of the TREtight inducible promoter and with an SV40 polyA tail, were sub-cloned into a targeting vector flanked with homology arms (~900 bp) targeting the ROSA26 locus. We used CRISPR-Cas9 to target the transgenes to the safe harbor mouse ROSA26 locus in a best-case scenario. Alternatively, should CRISPR-based homology-directed repair fail we hypothesized that, from the same pro-nuclear injections, random integration of the transgene would still generate viable and useful transgenic mouse models, albeit with unknown genomic integration site and copy number. Here we report that while these constructs can successfully integrate into the mouse genome, they are unstable and full-length insertions are not retained throughout generations.

2. Materials and methods

2.1. Constructs

We previously generated alternative codon constructs for expression of DPRs over 1000 repeats in length and are 70% GC rich (GA1020, GR1136, PR1100, and AP1024; Bennion Callister et al., 2016). DPR sequences from these constructs were cloned into a pUC57-ROSA26 Gateway targeting vector (provided by Dr Antony Adamson) under the control of a tetracycline-responsive promoter from the pTRE-Tight vector (ClonTech), to facilitate inducible DPR expression in mice. Due to the long, repetitive nature of the transgene, it was not possible to sequence the entire insert, and therefore sequencing was performed at each end of the repeat and the insert was digested out of the vector and run on an agarose gel to confirm repeat length. The entire transgene, including an SV40 polyA tail and flanking homology arms for targeted integration into the ROSA26 locus, was excised by restriction digest and purified by gel extraction using ultra-pure agarose and the Monarch Gel Extraction Kit (New England Biolabs), followed by a further column purification (Monarch PCR & DNA Cleanup Kit; New England Biolabs) and elution in injection buffer (Tris-HCl 1 mM, pH 7.5, EDTA 0.1 mM; Millipore).

2.2. Embryo microinjection

For mouse embryo microinjection, a crRNA oligo to target the ROSA26 locus (ACTCCAGTCTTTCTAGAAGA-*TGG*; Chu et al., 2016) was annealed with tracrRNA (Integrated DNA Technologies) by diluting in injection buffer (Millipore) and heating to 95 °C. Annealed oligo was complexed with Cas9 protein by mixing in a 1:1 ratio and incubating at room temperature for 10 minutes. Purified DNA template was added to a final concentration of 10 ng/μL (gRNA and Cas9 final concentration of 20 ng/μL), before brief centrifugation (5 minutes, 10,000 *g*). The supernatant was microinjected into C57BL/6J (Envigo, UK) zygote pronuclei using standard protocols (Nagy et al., 2006). Zygotes were cultured overnight and the

resulting 2 cell embryos surgically implanted into the oviduct of day 0.5 post coitum pseudopregnant C57BL/6J mice. For all 4 DPR injections we experienced normal levels of embryo survival and numbers of live births. All work adhered to the UK Animals (Scientific Procedures) Act 1986. Animals were kept under PPLs P08B76E2B and 70/08903.

2.3. Genotyping: polymerase chain reaction

Pups were ear punched after weaning and genomic DNA extracted for polymerase chain reaction (PCR) analysis using primers (universal for all 4 constructs; Table 1). PCR was performed using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). We also performed flanking PCRs from the genomic site found outside the homology arms to the unique primers in our targeting cassette but failed to detect targeted integration by CRISPR-Cas9 (not shown). Pups positive for either 5′ or 3′ PCRs were taken forward for genotyping by Southern blot to confirm integration of repeat elements.

2.4. Genotyping: Southern blotting

Tail samples were incubated in 0.5 mg/mL proteinase K overnight at 57 °C. DNA was then purified by phenol:chloroform extraction (Sambrook and Russell, 2006). Southern blotting was carried out using DIG-labeled probes specific to each DPR (Eurofins MWG; Table 2). Five micrograms of DNA was digested overnight with NlaIII and DdeI (New England Biolabs). Samples were electrophoresed in 0.8% agarose 1 × TBE gels run at 70 V for 3 hours. The agarose gels were depurinated in 0.25 M HCl for 10 minutes, denatured in 0.6 M NaCl, 0.2 M NaOH for 30 minutes, and neutralized in 1.5 M NaCl, 0.5 M Tris-HCl pH 8 for 30 minutes. DNA was transferred to a positively charged nylon membrane (GE Healthcare) by capillary blotting and crosslinked by UV irradiation. Membranes were pre-hybridized in DIG Easy Hyb buffer (Sigma Aldrich) with 100 μg/mL denatured salmon sperm DNA (Agilent) for 4 hours at 42 °C, after which the membrane was hybridized with 5 ng/μL DIG-labeled probe overnight at 42 °C in a rotating hybridization oven. The membrane was then washed once with 2 × SSC (saline sodium citrate), 0.1% SDS solution at room temperature for 1 minute and twice with 0.2 × SSC, 0.1% SDS solution at 65 °C for 15 minutes per wash. Membranes were equilibrated in maleic acid buffer (Sigma Aldrich) and incubated for 30 minutes in blocking solution (Sigma Aldrich) before being incubated with anti-DIG antibody (1:20,000, Sigma Aldrich) for 30 minutes and washed twice in wash buffer for 15 minutes per wash at room temperature. Membranes were equilibrated with detection buffer for 5 minutes at room temperature, and then incubated with CPD-Star solution (GE Healthcare) before imaging using a Syngene G-Box Gel Documentation System. A positive control (pure DPR-containing plasmid) and negative control (wild-type mouse DNA) were included on each blot. Bands were sized using the DIG-II or DIG-III DNA ladder (Sigma Aldrich). The number of mice generated and genotyped by Southern blot in each generation is shown in Table 3.

Table 1
Genotyping primers used universally for all 4 constructs

Insert end	Direction	Sequence
5′	Forward	TCTCCCAAAGTCGCTCTGAG
	Reverse	CCCTTGAACCTCCTCGTTCGACC
3′	Forward	GGGATCCTCTAGTCAGCTGACGCGTGC
	Reverse	GCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGA

Table 2
DIG-tagged probe sequences used for detection of DPR sequences by Southern blot

DPR	Probe sequence
GA probe	DIG-GGCAGGAGCTGGAGCTGGCGCAGGAGCTGGTGCTGGG-DIG
GR probe	DIG-AGGCAGAGGTCGTGGGAGAGGCAGGGGTCGCGGACGTGGA-DIG
AP probe	DIG-AGCACCAGCACCGGCGCCAGCTCCAGCACCAGCACCC-DIG
PR probe	DIG-AGACCACGTCCTAGGCCCAGACCCAGACCCAGGCCTA-DIG

Probes were tagged at both ends.
Key: DIG, digoxigenin; DPR, dipeptide repeat protein.

3. Results

3.1. GA1020

GA1020 integrated into the genome in the F0 generation at full length and animals survived to adulthood. Mice were screened using PCR assays detecting the 5′ and 3′ end of the insert and animals having either or both ends were genotyped using Southern blot (Fig. 1A). Full-length insert was detected at ∼6.6 kb (approximately 1000 repeats). Four GA1020-positive F0 mice were mated with C57BL6/J wild-type mice. Litters were small and 2 founders produced no live offspring. Founder 12 produced no mice positive for the insert suggesting that it had not integrated into the germline. Founder 14 produced 15 offsprings, of which 3 were positive in the PCR screen. However, when DNA from these mice was Southern blotted, GA insert was detected at ∼3 kb, approximately 600 DPR repeats (referred to as GA600), suggesting that the insert had shrunk between generations (Fig. 1B). Three GA600$^{+/-}$ mice were bred to wild-type animals. One mouse was culled due to birthing difficulties. Due to the unreliability of the PCR screen samples were taken from all offsprings and Southern blotted. No samples were positive for GA DNA at any size (Fig. 1C and D).

3.2. GR1136

GR1136 also integrated into the genome in the F0 generation at full length and animals survived to adulthood. Full-length insert was detected at ∼6.4 kb (approximately 1000 repeats) (Fig. 2A). Both animals identified as positive by Southern blot were mated with wild-type mice. Founder 7 produced 1 litter of which 1 mouse screened positive for full-length GR1136 insert (Fig. 2B). Founder 21 produced 7 offsprings and, similar to the GA strain, GR probe was able to detect an insert of reduced size (approximately 500 repeats; GR500) in 3 animals (Fig. 2C). In all cases, a positive PCR screen for either end of the insert did not predict a positive Southern blot sample. Neither the single GR1136$^{+/-}$ mouse nor the 3 GR500$^{+/-}$ mice produced GR-positive offspring (Fig. 2D–F).

3.3. AP1024 and PR1100

AP1024 only integrated into the F0 generation at a shortened repeat size of approximately 400 repeats (Fig. 3A). However, when these founders were bred forwards the AP transgene had not

Table 3
Number of mice generated and genotyped by Southern blot in each generation

DPR	F0		F1		F2	
	Total	Blotted	Total	Blotted	Total	Blotted
GA1020	30	9	3	3	32	32
GR1136	27	4	8	8	49	49
PR1100	27	16	NA	NA	NA	NA
AP1024	31	5	10	10	NA	NA

Key: DPR, dipeptide repeat protein; NA, not applicable.

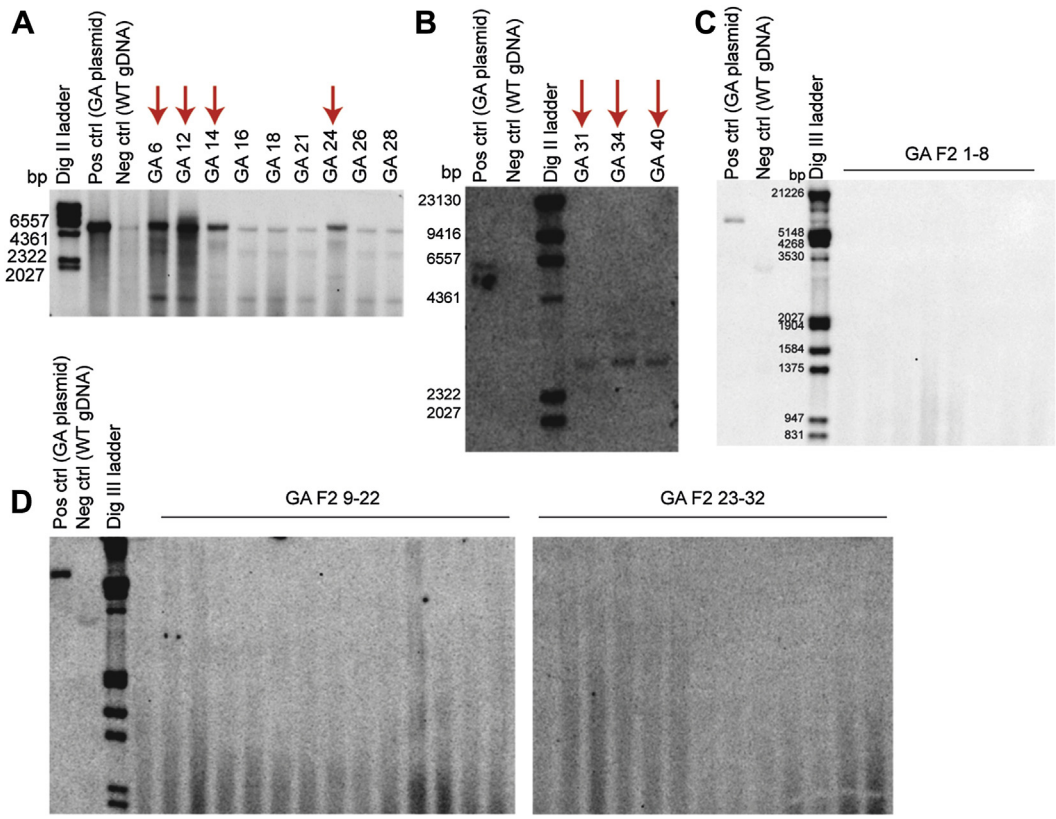

Fig. 1. GA1020 inserted at full length but was not maintained over generations. (A) In the F0 generation, full-length GA was detected at ~6.6 kb (~1000 repeats) in founders GA6, 12, 14, and 24. Several smaller bands were also present, indicating instability of the repeat within individual animals. (B) GA DNA was detected at ~3 kb in 3 offsprings from founder 14. (C and D) No samples from F2 were positive for GA DNA at any size in the Southern blot. Arrows indicate that animals were bred forwards. GA, glycine-arginine.

integrated into the germline in any animals as shown by failure to detect any AP repeat DNA in the F1 generation (Fig. 3B). Although multiple animals were identified as positive for PR1100 by PCR assays, none tested positive in the Southern blot (Fig. 3C).

3.4. Correlation between PCR genotyping and Southern blot varied between strains

The correlation between positive PCR genotyping and detection of a DPR insert by Southern blot is described in Table 4. In both GA and GR strains, only half of positive PCR samples were then positive in Southern Blot. For PR, none of the samples that tested positive in the PCR proved to have any insert at all. On the other hand, all PCR-positive AP1024 samples were also positive in the Southern Blot, albeit at a reduced insert length. Taken together this suggests that PCR genotyping is not reliable in identifying mice with transgenic DPR inserts.

4. Discussion

Current mouse models of the C9orf72 expansion vary widely in phenotype and neuropathology and many cannot distinguish between the effects of RNA foci and DPRs (Chew et al., 2015; Jiang et al., 2016; Liu et al., 2016; O'Rourke et al., 2015; Peters et al., 2015). There are several studies of individual DPR species which suggest that specific DPRs may have different effects on neurodegeneration and behavioral and cognitive defects (Choi et al., 2019; Hao et al., 2019; Schludi et al., 2017; Zhang et al., 2016, 2018, 2019). However, given our previous finding that DPR toxicity is determined by repeat length (Bennion Callister et al., 2016; Swaminathan et al., 2018), mouse models expressing DPRs at repeat lengths found in patients are urgently needed. Our proposed model would have allowed us to analyze and compare the effect of each DPR at disease-relevant lengths on pathology, neurodegeneration, and behavioral phenotypes over time in an inducible, tissue-specific manner.

We have shown that it is possible to use CRISPR to produce knock-in mice with DNA coding for DPRs of a physiologically relevant size, but that using alternative codons to code for DPRs does not protect the DNA from excision or truncation between generations. It is well-known that tandem repeats are unstable in DNA plasmid vectors (Thapana et al., 2014), artificial chromosomes (Neil et al., 1990; Song et al., 2001), cell lines (Pelletier et al., 2005), and mouse models (Al-Mahdawi et al., 2004; Gazy et al., 2019; Gourdon et al., 1997; Zhao et al., 2015). Double-strand breaks (DSBs) are a potential source of this instability, particularly during meiosis when multiple DSBs are induced into the genome during chromatin remodeling (Cohen et al., 1999; Simard et al., 2014). This may be the point at which our DPR inserts lose size due to replication slipped misalignment, mismatch, or DSB repair. It has been shown that C9orf72 repeat size in patients varies hugely between different tissues through somatic mosaicism and repeat instability (Gijselinck et al., 2016; van Blitterswijk et al., 2013), and it has also been reported that repeat lengths can contract between generations (Gijselinck et al., 2016). A recent study also found that repeat lengths can expand and contract in the same patient and the same tissue over time, with multiple blood samples yielding different repeat-length measurements (Fournier et al., 2018).

Although we hoped that reducing the repetitive nature and GC content of constructs through use of alternative codons to those

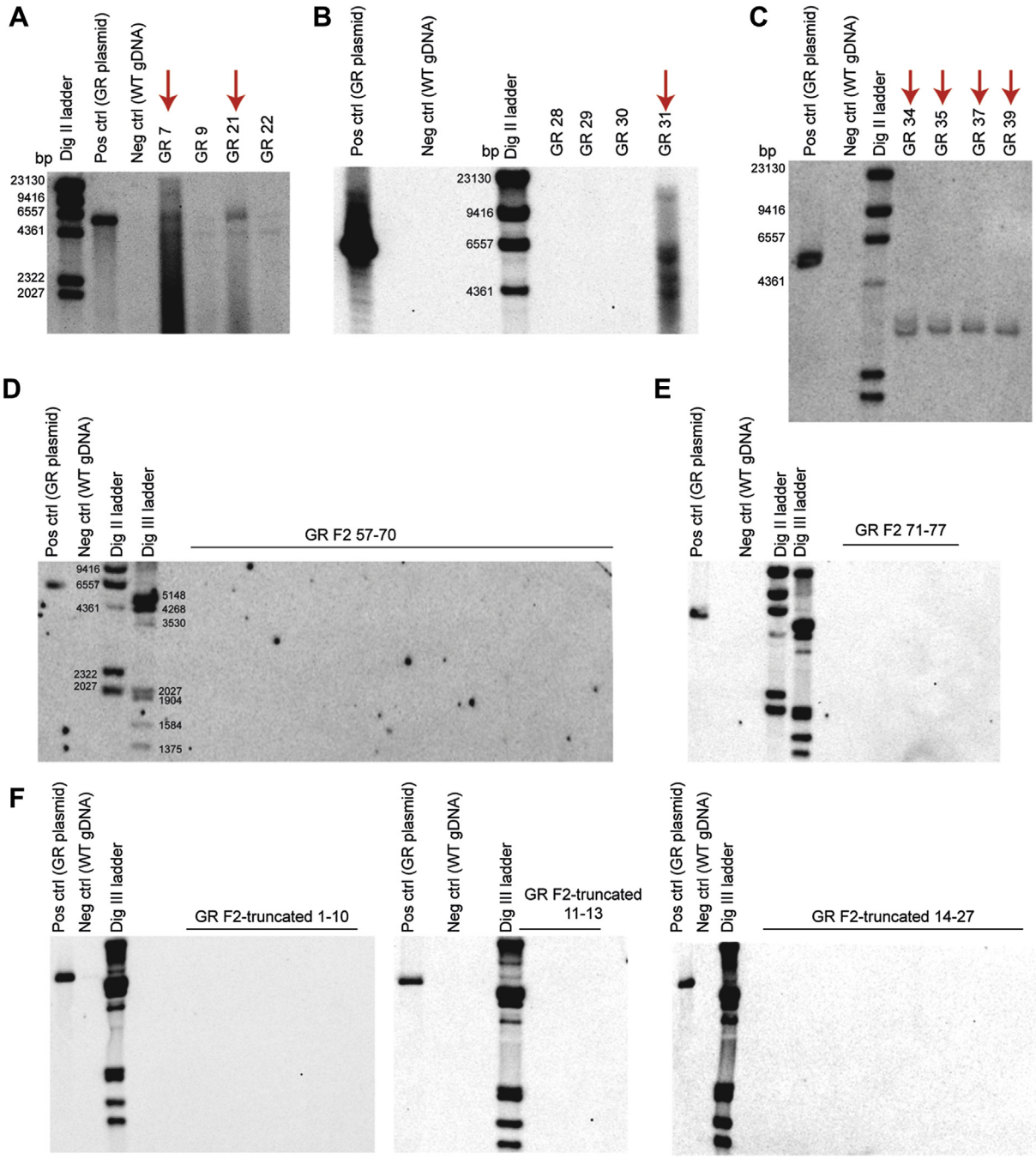

Fig. 2. GR1136 inserted at full length but was not maintained over generations. (A) In the F0 founder generation, full-length GR insert was detected at ~6.4 kb (approximately 1000 repeats) in founders GR7 and 21. (B) Full-length GR DNA was detected in 1 mouse from founder 7. (C) GR DNA was detected at ~3 kb in 4 offsprings from founder 21. (D and F) No samples from F2 were positive for GR DNA at any size in the Southern blot. Arrows indicate that animals were bred forwards. The presence of multiple bands indicates instability of the repeat length within individual animals in both the F0 and F1 generations.

found in the expansion would protect from instability, it appears that this is not sufficient. Several of the Southern blots showed multiple bands indicating that DPR sequences were present at multiple repeat lengths within a single animal, demonstrating instability of the inserts even before excision in later generations. Existing BAC mice expressing pure G_4C_2 repeats within the human C9orf72 gene have been slightly more successful at retaining repeat length across generations, perhaps because these transgenes contain large regions of flanking DNA which could protect the repeat sequence from excision. It is possible that the C9orf72 gene contains an endogenous sequence which protects from excision between generations, and that this element is retained when expressing repeats within the wild-type gene sequence. However, published reports of these BAC mice also indicate a degree of instability in repeat length between individual mice in each cohort.

Furthermore, we found that PCR assays detecting the ends of transgenic DNA gave false positive results and therefore are not a

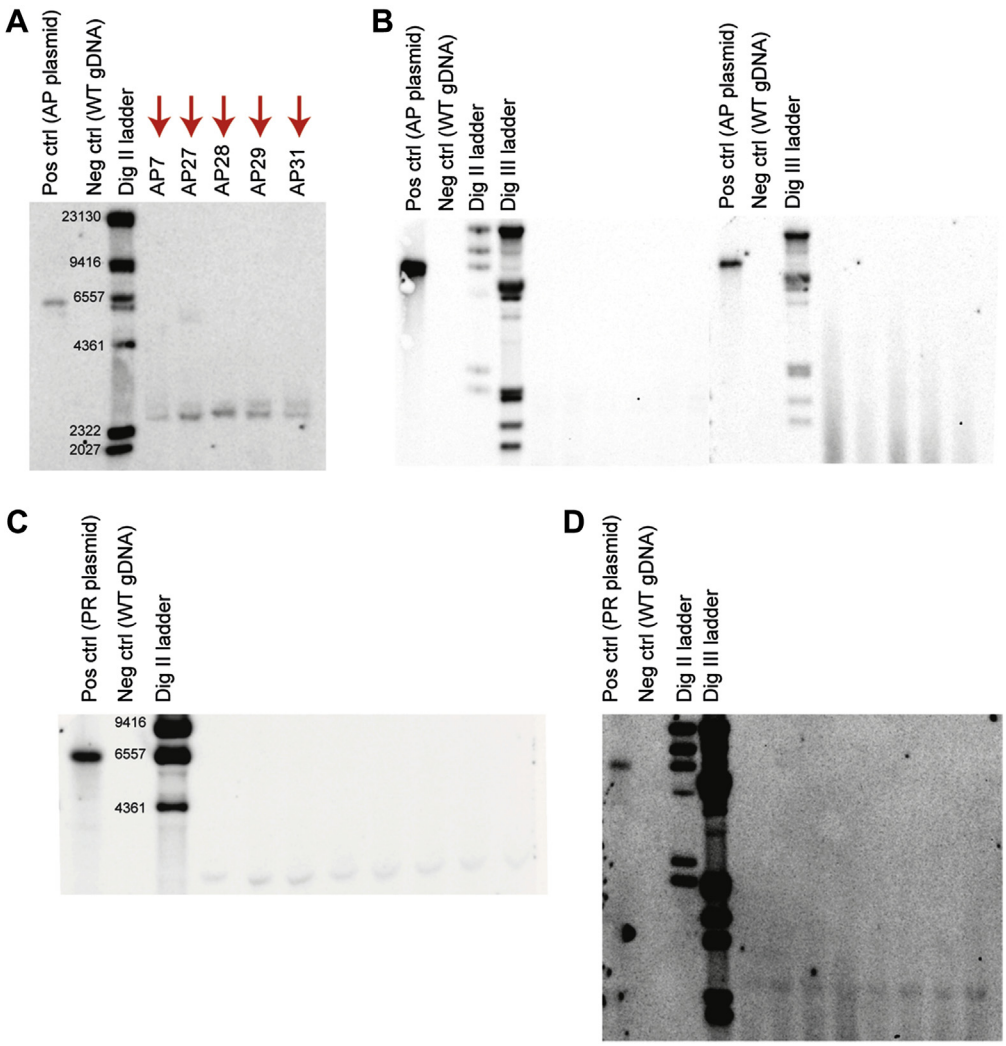

Fig. 3. AP1024 inserted at a truncated length and was not maintained, while PR1100 did not insert into the genome. (A) AP1024 only integrated into the F0 generation at a shortened repeat size of approximately 400 repeats. Two distinct bands indicate instability of the repeat length within individual animals. (B) No offspring from any of AP F0 mice was positive for AP DNA at any size. (C and D) PR1100 did not integrate into the mouse genome.

reliable method of confirming maintenance of the DPRs in a colony. Particularly in the case of PR1100, we found that multiple samples were positive in PCR screens while none of these were positive in the Southern blot. This may be caused by instability of the repetitive element removing the DPR region but retaining the 5′ or 3′ end of the transgene. The unreliable nature of the PCR assays highlights the importance of genotyping all animals by Southern blotting when modeling large repeat expansions.

Table 4
Comparison of PCR-positive samples and SB-positive results

DPR	Percentage of animals with positive PCR	Percentage of animals with positive SB	Percentage of PCR-positive animals also positive on SB
GA1020	25.53	14.89	58.33
GR1136	35.00	17.50	50.00
PR1100	59.26	0.00	0.00
AP1024	16.13	16.13	100.00

Only half of PCR-positive animals from GA1020 and GR1136 lines were also positive in SB. No PR1100 animals were SB-positive, whereas all AP1024 PCR-positive animals were also positive in SB.
Key: DPR, dipeptide repeat protein; PCR, polymerase chain reaction; SB, Southern blot.

5. Conclusions

Stable integration of long DPR sequences into the mouse genome using CRISPR-Cas9 was unsuccessful, with shrinkage or complete removal of repeat sequences occurring between generations. Mouse models of C9FTD/ALS currently in use have variable repeat lengths and most do not recapitulate the repeat lengths found in patients. Future work will need to address this issue to produce novel models of full-length DPR pathology in mammals. AAV administration of long DPR constructs after birth could be a more suitable method for generation of such models, as reported previously for much shorter DPR repeats (Choi et al., 2019; Schludi et al., 2017; Zhang et al., 2016, 2018, 2019). However, stable integration of long DPR sequences in mice may not be possible using current technology.

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