Cell-type-specific dysregulation of RNA alternative splicing in short tandem repeat mouse knockin models of myotonic dystrophy

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Short tandem repeats (STRs) are prone to expansion mutations that cause multiple hereditary neurological and neuromuscular diseases. To study pathomechanisms using mouse models that recapitulate the tissue specificity and developmental timing of an STR expansion gene, we used rolling circle amplification and CRISPR/Cas9-mediated genome editing to generate Dmpk CTG expansion (CTGexp) knockin models of myotonic dystrophy type 1 (DM1). We demonstrate that skeletal muscle myoblasts and brain choroid plexus epithelial cells are particularly susceptible to Dmpk CTGexp mutations and RNA missplicing. Our results implicate dysregulation of muscle regeneration and cerebrospinal fluid homeostasis as early pathogenic events in DM1.

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Microsatellites, or short tandem repeats (STRs) of ≤10 bp are unstable genetic elements with a propensity to fold into intrasstrand DNA structures that compromise replication, recombination, and repair pathways (Usdin et al. 2015; Neil et al. 2017). While STRs are susceptible to both expansions and contractions, expanded STRs cause >40 neurological and neuromuscular hereditary diseases, including C9orf72 amyotrophic lateral sclerosis and frontotemporal dementia [C9-ALS/FTD], Huntington disease [HD] and myotonic dystrophy types 1 [DM1] and 2 [DM2] (Nelson et al. 2013). DM1 is a prominent STR disease as it is the most common adult-onset muscular dystrophy and is caused by CTG expansions [CTGexp] in the 3′ untranslated region [3′UTR] of the DMPK gene. Repeat sizes range from 5 to 37 in unaffected individuals but 50 to >4000 in DM1 patients (Goodwin and Swanson 2014). The DM1 pathomechanism involves transcription of expanded DMPK alleles, which generates toxic CUGexp RNAs that accumulate in nuclear inclusions or RNA foci, where they sequester muscleblind-like (MBNL) proteins. Since MBNL proteins regulate the splicing of their RNA targets during development, loss of MBNL function results in the expression of developmentally inappropriate isoforms in multiple tissues (Thomas et al. 2017).

Attempts at modeling DM1 in mice have relied on heterologous promoters and gene contexts, which disconnects the tissue specificity, developmental timing, and spatial expression of a repeat expansion from its endogenous gene context. This dissociation could mask discoveries that might have important pathomechanistic implications. Here, we use a combination of rolling circle amplification (RCA) and CRISPR/Cas9 genome editing to introduce CTG expansions into the mouse Dmpk 3′UTR. Our results highlight important interplays between Dmpk and MBNL expression patterns with CTGexp length and missplicing, and also demonstrate that splicing regulation in the choroid plexus, which is responsible for the bulk of cerebral spinal fluid (CSF) production, is adversely affected by CTGexp mutations.

Results and discussion

Dmpk CTGexp knockin mice

Since DMPK exons are well conserved among mammals, with the exception of an uninterrupted CTG repeat tract in the human 3′UTR [Supplemental Fig. S1A], we developed a microsatellite expansion modeling by RCA and genome editing (MERGE) protocol to insert CTGexp mutations into the corresponding region of the mouse Dmpk 3′UTR [see Supplemental Material]. Target site selection was validated in C2C12 myoblasts using multiple guide [g]RNAs followed by determination of cleavage efficiencies [Supplemental Fig. S1B; Ran et al. 2013]. After establishing the optimal site, adjacent sequences were cloned flanking the CTGexp repeat track as arms of homology [Fig. 1A]. The resulting plasmid was amplified by RCA to maintain repeat length, and the resulting DNA served as a template for homology-directed repair (HDR) and for generation of larger repeat templates in vitro using Golden Gate Assembly (Osborne and Thornton 2008). Two rounds of Golden Gate Assembly were performed, with each cycle achieving a repeat content of 2n-2, where n is the starting CTG202 repeat size, resulting in CTG402 and CTG802 HDR templates [Fig. 1B]. As proof of concept for this approach, we first generated mouse Dmpk knockin [KI] lines using CRISPR/Cas9 with a CTGexp HDR template. Of 74 newborn pups, four were positive for Dmpk insertion as analyzed by PCR using a forward primer outside the homology arm [Supplemental Fig. S1C]. Interestingly, Dmpk CTGexp recombination...
CTGexp mutations induce DMPK protein loss in skeletal muscle and myotubes

Since skeletal muscle weakness/wasting and myotonia are characteristic features of DM1, and Dmpk expression is relatively high in this tissue [Supplemental Fig. S2A], we initially assessed the impact of CTGexp mutations on Dmpk CTGexp KI muscle. CUGexp RNA foci were detectable by RNA fluorescence in situ hybridization [RNA-FISH] in adult skeletal muscles of Dmpk CTGexp mice [Supplemental Fig. S2B], so we tested whether CTGexp insertions altered Dmpk expression in tibialis anterior [TA] muscles from Dmpk/170 and Dmpk/170/170 mice by RT-qPCR. Interestingly, Dmpk RNA levels were affected by these mutant alleles and the total number of CTG repeats [Supplemental Fig. S2C]. While this apparent RNA decrease might reflect decreased Dmpk transcription and/or increased Dmpk RNA turnover, previous cell DM1 model studies have shown that the isolation of CUGexp RNAs is impaired using traditional RNA extraction methodologies, possibly due to nuclear retention in RNA foci [Davis et al. 1997]. The presence of CUGexp RNA foci and reduced Dmpk RNA levels made us question whether the production of DMPK protein was also altered. Indeed, when assessed by immunoblot analysis we detected a dramatic decrease in DMPK protein levels that was inversely correlated to the number of mutant CTG170 alleles [Supplemental Fig. S2D]. DMPK protein levels in the TA from Dmpk/170 mice decreased by ∼50%, while levels in Dmpk/170/170 dropped >90%. These results demonstrated that even a moderate-sized CTG repeat expansion in a mouse Dmpk KI mutant severely compromised protein production from the mutant allele in agreement with early studies that showed decreased expression of DMPK protein in DM1 [Fu et al. 1993]. Importantly, we did not detect abnormal muscle phenotypes, including myotonia and centralized myonuclei, in either Dmpk CTG170 or CTG480 heterozygous or homozygous mice [data not shown]. This result was consistent with earlier studies on Dmpk knockout mice, which fail to replicate DM-relevant phenotypes and only develop a very mild late-onset myopathy [Jansen et al. 1996; Reddy et al. 1996].

Although RNA-foci were present in Dmpk CTGexp KI mice, the lack of other DM1-associated muscle phenotypes suggested that alternative splicing regulation was not severely altered in this tissue. Indeed, RNAseq followed by RT-PCR validations failed to identify significant changes in DM1-relevant alternative splicing events [Supplemental Table S1; Supplemental Fig. S2E]. For example, missplicing of Atp2a1 exon 22 and Clcn1 exon 7a, which are misregulated in DM1 muscle, was not detectable in Dmpk CTG170 or CTG480 KI mouse in contrast to Mbnl1−/− knockout TA muscle [Supplemental Fig. S2E]. Furthermore, differential gene expression analysis using DESeq2 did not identify major [greater than two-fold] changes in gene expression [Supplemental Fig. S2F].

The lack of overt muscle and splicing phenotypes was surprising since our Dmpk KI mutants expressed alleles that are considered to be pathogenic in humans. This result suggested that the temporal and spatial expression of a stable expansion in the Dmpk gene in skeletal muscle did not produce a sufficient CUG repeat load to drive pathology. Based on our earlier finding that human myotubes have higher DMPK expression than adult muscle [Thomas et al. 2017], we tested the possibility that CTGexp mutations might have more severe effects on.

Figure 1. Generation of Dmpk knockin mice carrying CTG 3′ UTR expansions. [A] CRISPR/Cas9-mediated recombination of HDR templates containing a CTG expansion flanked by homology arms at the Dmpk 3′ UTR. External [blue] and internal [red] hybridization probes are indicated together with forward [F1] and reverse [R1] PCR primers. [B] Repeat dimerization by Golden Gate Assembly followed by RCA. Plasmids containing a repeat expansion flanked by Bsal and BbsI were linearized by Sapl, linear constructs were separately digested by Bsal or BbsI, and repeat-containing fragments were gel purified, ligated, and amplified by RCA. This process generates a new construct carrying 2n-2 repeats, where “n” is the initial expansion size. [C] PCR of CTG170, CTG480, and CTG602 HDR templates. Linearized RCA products yielded a 2-kb backbone band [bb] and upper bands corresponding to the recombination template [dashed white lines indicate the isolated HDR templates]. [D] PCR genotyping of Dmpk+/+ [wild-type], Dmpk+/170, and Dmpk+/480 mice.
muscle precursor cells, including primary myoblasts. In agreement with this idea, CUG\textsuperscript{exp} RNA foci were considerably more numerous in myoblasts and differentiated myotubes compared with adult skeletal muscle and were particularly striking in the Dmpk\textsuperscript{480} heterozygous and homozygous myotubes (Fig. 2A). CUG\textsuperscript{exp} RNA foci number increased upon myogenic differentiation, in agreement with the temporal up-regulation in number increased upon myogenic differentiation, in agreement with this idea, CUG\textsuperscript{exp} RNA foci were considerably more numerous in myoblasts and differentiated myotubes (Supplemental Fig. S2C), CTG\textsuperscript{exp} mutations led to a decrease in the amount of RNA isolated from both myoblasts and myotubes (Fig. 2B). As suggested above, this was due to an RNA extractability issue since Trizol extraction at an elevated temperature (55°C), a procedure shown to release nuclear-retained RNAs (Chujo et al. 2017), resulted in increased Dmpk RNA levels isolated from KI cells (Fig. 2B; Supplemental Fig. S2G). Myotube DMPK protein levels decreased similarly to Dmpk CTG\textsuperscript{exp} skeletal muscle with no detectable DMPK protein in Dmpk\textsuperscript{480/480} myotubes (Fig. 2C; Supplemental Fig. S2H).

MBNL sequestration and RNA missplicing in Dmpk CTG\textsuperscript{exp} myotubes

Misregulation of alternative splicing in DM1 results from MBNL sequestration by CUG\textsuperscript{exp} RNA so we next determined whether MBNL proteins were effectively sequestered in nuclear RNA foci of differentiated Dmpk CTG\textsuperscript{exp} KI myotubes. Confocal immunofluorescence microscopy showed both MBNL1 and MBNL2 were localized throughout the nucleoplasm in wild-type (Dmpk\textsuperscript{+/+}) cells, but clearly relocalized to foci in CTG\textsuperscript{exp} myotubes (Supplemental Fig. S3A). In contrast, the localization of another nuclear RNA-binding protein, HNRNPH1, was unaffected. RNA-FISH and IF confirmed that MBNL1 was sequestered in CUG\textsuperscript{exp} RNA foci (Supplemental Fig. S3B), which had a punctate distribution pattern by super-resolution microscopy (Supplemental Fig. S3C). Since MBNL proteins were sequestered in nuclear RNA foci and substantially depleted from the nucleoplasm pool, we next determined whether alternative splicing changes occurred in Dmpk CTG\textsuperscript{exp} myotubes.

RNAs were isolated from Dmpk\textsuperscript{+/+}, Dmpk\textsuperscript{170/170}, Dmpk\textsuperscript{480/480}, and Dmpk\textsuperscript{480/480} myotubes for RNA-seq analysis. Similar to skeletal muscle, differential gene expression analysis showed only subtle changes due to CTG\textsuperscript{exp} mutations [Supplemental Fig. S2F]. In contrast to TA muscle, alternative splicing was significantly dysregulated and the number of missplicing events correlated with CTG repeat load with 243 events in Dmpk\textsuperscript{480/480} but only 110 in Dmpk\textsuperscript{+/+} and 19 in Dmpk\textsuperscript{170/170} myotubes (Fig. 3A; Supplemental Table S2). Many of the top alternative splicing (AS) changes (Cacna1s exon 29, Cacna2d1 exon 19, Ncor2 exon 46 5′ss, Pdlim3 exon 4 5′ss, and MBNL1 exon 5) have been previously characterized in DM1 cells and mouse models (Kimura et al. 2005; Tang et al. 2012; Lee et al. 2013). A prior study proposed 46 AS events as specific biomarkers for DM1 muscle (Wagner et al. 2016) with 26 of these events conserved in mice. Over 40% of these events [11/26] were present in our Dmpk\textsuperscript{480/480} myotube data set with changes in Cacna1s, Cacna2d1, Clasp1, Dnm1, Mbnl1, Mxra7, Nfix, Pdlim3, Pdlim3.2, Slain2, and Sorbs1. Selected CUG\textsuperscript{exp}-induced splicing shifts were validated by RT-PCR from separate differentiation experiments using three independent cell lines, and these expansion-induced splicing changes were MBNL dependent since they were similarly dysregulated in Mbnl1\textsuperscript{−/−} knockout myotubes (Fig. 3B; Supplemental Fig. S3D). For example, increased selection of a distal alternative 5′ splice site (ss) in Ncor2 exon 46 occurred with higher CTG\textsuperscript{exp} repeat load, while there was a switch in Ralgapa1 from a more distal to a more proximal 5′ss that resulted in a truncated exon 17 (Supplemental Fig. S3D). Interestingly, we also detected a previously uncharacterized AS change for Dmpk with decreased use of an exon 14 alternative 3′ss in CTG\textsuperscript{exp} myotubes compared with WT [Supplemental Fig. S3E]. To clarify why myoblasts and myotubes showed RNA foci and RNA processing changes while skeletal muscle did not, we tested whether Dmpk RNA levels exceeded Mbnl in cells and tissues prone to CUG\textsuperscript{exp}-induced missplicing. Indeed, the Dmpk/Mbnl RNA ratio was much higher in myoblasts and myotubes compared with all skeletal

Figure 2. RNA foci and DMPK protein loss in CTG\textsuperscript{exp} KI primary myoblasts and myotubes. (A) RNA-FISH of Dmpk WT (Dmpk\textsuperscript{+/+}), compared with Dmpk CTG\textsuperscript{exp} heterozygous and Dmpk CTG\textsuperscript{exp} homozygous primary myoblasts and differentiated myotubes, which show numerous nuclear CUG repeat expansion (CUG\textsuperscript{exp}) RNA foci [red] in the nucleus [blue, DAPI]. Scale bars = 10 µM. (B) Reduced isolation of myoblast Dmpk CUG\textsuperscript{exp} [heterozygous Dmpk CTG\textsuperscript{exp}, homozygous CTG\textsuperscript{exp/480}] RNAs at 25°C [blue bars] is prevented by high temperature [red, 55°C] Trizol extraction ([**] P < 0.05, [***] P < 0.01, [****] P < 0.0001, one-way ANOVA). (C) Inhibition of Dmpk mRNA nuclear export in cells expressing Dmpk CTG\textsuperscript{exp} genes [Dmpk CTG\textsuperscript{170/170}, Dmpk CTG\textsuperscript{480}], Dmpk CTG\textsuperscript{480/480}] leads to decreases in DMPK protein expression that correlate with CTG repeat length. [***] P < 0.01; [****] P < 0.001; [*****] P < 0.0001; one-way ANOVA.

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muscle types examined, suggesting that MBNL activity might be more effectively sequestered in myoblasts and myotubes compared with muscle [Supplemental Fig. S3F]. Overall, these results suggested that tissue and developmental-stage specific missplicing in DM1 reflects the interplay between repeat load, including repeat expansion length and host gene expression, in combination with MBNL protein levels. To test this possibility, we reduced MBNL1 levels by generating Mbnl1−/−; Dmpk480/480 mice. As expected, significant DM1-associated missplicing events were not detected in Dmpk480/480 or Mbnl1−/−, but were significantly dysregulated in Mbnl1−/−; Dmpk480/480 muscle (Fig. 3C).

We next tested whether Dmpk CTGexp KI myoblasts were a suitable cell model for testing drugs to correct AS changes in DM1. A recent study reported that furamidine, an analog of the antimicrobial pentamidine, corrected missplicing in both DM1 HeLa cell and HSA GR mouse models possibly by promiscuous binding interactions with both DNA CTG · CAG and RNA CUG repeats with nM affinity as well as disrupting MBNL1-CUG complexes at μM concentrations [Jenquin et al. 2018]. Myoblasts were differentiated for 24 h prior to treatment with furamidine for 48 h, followed by RNA isolation and RT-qPCR to assess the rescue of top AS changes observed in KI myotubes. All furamidine doses showed a shift toward the WT AS pattern and 2 μM was the most effective with ≥50% rescue of two of the top AS events [Supplemental Fig. S3G]. This data confirmed that Dmpk CTGexp KI myoblasts provide tractable cell models for screening and assessment of small molecule drugs.

**CTGexp mutations induce choroid plexus spliceopathy**

While DM1 is classified as a muscular dystrophy, this is a multisystemic disorder in which the CNS is profoundly affected by hypersomnia, cognitive and behavioral abnormalities, progressive memory deficits, cerebral atrophy, and missplicing of MBNL2 target RNAs [Goodwin and Swanson 2014; Meola and Cardani 2015]. One of the strengths of our CTGexp KI approach is that repeat expansion expression is driven by the endogenous Dmpk gene, which allows an unbiased screen for cell populations affected in vivo. Since myoblasts were strikingly affected by expression of CTGexp RNAs, we were interested in whether a specific cell type was more susceptible to dysregulation in the brain. Thus, we assembled a panel of multiple CNS regions and performed RT-qPCR to determine Dmpk RNA levels. Although most brain regions had very low expression, Dmpk was highly expressed in the choroid plexus [ChP] with levels exceeding skeletal muscle [Supplemental Figs. S2A, S3F]. The ChP is responsible for the majority of cerebral spinal fluid (CSF) production and is composed of epithelial cells surrounding a fenestrated capillary network that protrudes from the brain parenchyma into the ventricular lumen [Lun et al. 2015]. Moreover, DMPK protein levels are particularly high in the apical region of these cells [Whiting et al. 1995].

RNA-FISH of Dmpk CTGexp KI brains revealed numerous CUGexp RNA foci in ChP (Fig. 4A) but less in other brain regions including hippocampus [Supplemental Fig. S4A]. RNA-seq analysis of isolated ChP [Fig. 4B] identified many missplicing events [Fig. 4C; Supplemental Table S3] in the absence of significant gene expression changes [Supplemental Fig. S4B]. Many of the top AS events were validated by RT-PCR [Fig. 4D; Supplemental Fig. S4C] and these AS changes in Dmpk CTGexp KI mice ChP were MBNL dependent since they were similarly dysregulated in Mbnl2−/− knockout ChP. These observations demonstrate that a key cell population important for CSF production accumulates RNA foci that compromise MBNL-mediated splicing activity, which could negatively impact CSF homeostasis and composition.

Dmpk CTGexp knockins reveal cell-specific susceptibility to STR expansions

While somatic mosaicism due to the expansion of CTG repeats is a characteristic feature of DM1, it is unclear whether these increases in repeat length determine
relevant muscle pathology, a result that agrees with previous human studies that detect CTG repeats up to 13-fold greater in DM1 muscle compared with repeat numbers in blood leukocytes (Thorton et al. 1994).

Another finding reported here with potential ramifications for our understanding of DM1 pathomechanisms is that the ChP is affected by alternative splicing deficits prior to skeletal muscle. The role of the ChP in CSF production involves selective transport so it is significant that several alternative splicing changes in Dmpk CTG<sup>exp</sup> KI mice ChP are involved in calcium and glutamate transport. Although the physiological outcome of these splicing changes in ChP is currently unclear, DM1 patients are affected by increases in ventricular volume and hyperomnia, which could be influenced by CSF homeostasis (Krogias et al. 2015; Meola and Cardani 2015). Future studies are required to determine whether Dmpk CTG<sup>exp</sup> KI mice have abnormal sleep patterns similar to Mbnl<sup>−/−</sup> knockout mice and whether DM patients have altered CSF content attributable to missplicing of ChP epithelial cell transport genes.

**Materials and methods**

**DMPK CTG<sup>exp</sup> knockin mice**

Optimal CRISPR/Cas9 Dmpk 3’UTR targeting sites were identified [http://crispr.mit.edu] and crRNA, tracrRNA, and SpCas9 protein (IDT) were transfected into C2C12 cells as an RNP complex using RNAiMAX (ThermoFisher) [Supplemental Table S4]. Genomic DNA was isolated and T7E1 assays (Alt-R Genome Editing Detection kit, IDT) were performed following the manufacturer’s instructions. Homology arms flanking the Dmpk cleavage site were PCR amplified and cloned into a CTG<sup>202</sup> plasmid (Dr. Charles Thornton, University of Rochester) by Gibson Assembly [New England Biolabs, NEB]. RCA was performed using 100 ng of CTG<sup>202</sup> annealed to 100 pmols of random hexamers (ThermoFisher) in 0.45× T4 DNA ligase buffer in a total volume of 22 µL. Next, 5 µL of 10µg/mL BsaI plasmid, 3 µL of 10 mM dNTP solution mix, 0.6 µL of 10 mg/mL BSA, 0.4 µL of yeast inorganic pyrophosphatase, and 1 µL of phi29 DNA polymerase [NEB] were added, followed by incubation at 37°C for 18 h followed by polymerase inactivation (65°C, 10 min).

To expand repeats, RCA products were separately digested by SapI-BsaI or SapI-BbsI, and the repeat-containing fragments were gel extracted, purified using AMPure XP beads [Beckman Coulter], and ligated overnight at 16°C using T4 DNA ligase [NEB]. Ligation products containing 2n-2 or SapI-BbsI, and the repeat-containing fragments were gel extracted, purified using AMPure XP beads (Beckman Coulter), and ligated overnight at 16°C using T4 DNA ligase [NEB]. Ligation products containing 2n-2 or SapI-BbsI, and the repeat-containing fragments were gel extracted, purified using AMPure XP beads. Linear recombination templates, together with sgRNA and Cas9 protein (System Biosciences), were microinjected into C57BL/6J zygotes (Harvard University Genome Modification Facility). Tail snips were genotyped by PCR and repeat-primed PCR (ABI3730 DNA Analyzer, Applied Biosystems) was performed to test for Dmpk CTG insertions. Results were processed using Gene Marker software (SoftGenetics). Southern blot analysis included internal and external probes to ensure single integrations at the expected site. Dmpk CTG<sup>170</sup> C57BL/6J mice were backcrossed to minimize potential unlinked Cas9-induced mutations, whereas CTG<sup>480</sup> BL6 males showed impaired mutant allele transmission so these mutants were outcrossed onto FVB/NJ. All animal procedures were reviewed and approved by the University of Florida IACUC. All primers and oligonucleotides are listed in Supplemental Table S4.

**Myoblast isolation, differentiation, and furamidine treatment**

Primary myoblasts were isolated and treated as described [see Supplemental Material].

**RNA-seq**

Total RNA was quality checked using a Fragment Analyzer, depleted of rRNA and cDNA libraries prepared [UltraII Directional RNA Library for future experiments].
Prep kit, NEBL. Tibialis anterior and myotube libraries used 500 ng, where-as choroid plexus libraries used 200 ng of total RNA. Sequencing was performed on the NextSeq500 Illumina platform. Fastq files were inspected using Fastqc. Differential gene expression studies used DESeq2 (Love et al. 2014). For splicing analysis, reads were aligned to the mouse genome [mm10] using STAR(v2.6.0a) (Dobin et al. 2013) and splicing was quantified using mATTS(v4.0.2) [Shen et al. 2012]. The mATTS output tables were filtered with the R package maser, with cutoff criteria of average reads ≥ FDR <0.05, and minimum change in splicing of 10% (|ΔΨ| ≥ 0.1). RNA-seq data sets are deposited in GEO under accession no. GSE137494.

Statistical analysis

Statistical significance was determined in GraphPad Prism by ordinary statistical analysis ≥ Jansen G, Groenen PJ, Bächner D, Jap PH, Coerwinkel M, Oerlemans F, van den Broek W, Gohlsch B, Pette D, Plomp JJ, et al. 1996. Abnormal myo-


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Author contributions: C.A.N., J.L.B., R.O., and M.S.S. designed the project, R.O. generated Dmpk CTG™ lines, C.A.N., B.M.K., R.O., and B.S.P. performed RNA-FISH and IF; C.A.N. and E.A.V. conducted super-resolution microscopy; C.A.N., J.L.B., B.A.O., L.J.S., H.A.C., and E.T.W. generated RNA-seq and RT-PCR results; R.O. and F.I. performed Southern blot analysis; C.A.N., J.L.B., R.O., and M.S.S. wrote the manuscript.

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