RESEARCH ARTICLE

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Altered RNA splicing contributes to skeletal muscle pathology in Kennedy disease knock-in mice

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SUMMARY

Here, we used a mouse model of Kennedy disease, a degenerative disorder caused by an expanded CAG repeat in the androgen receptor (*AR*) gene, to explore pathways leading to cellular dysfunction. We demonstrate that male mice containing a targeted *Ar* allele with 113 CAG repeats (AR113Q mice) exhibit hormone- and glutamine length-dependent missplicing of *Clcn1* RNA in skeletal muscle. Changes in RNA splicing are associated with increased expression of the RNA-binding protein CUGBP1. Furthermore, we show that skeletal muscle denervation in the absence of a repeat expansion leads to increased CUGBP1 expression. However, this induction of CUGBP1 is not sufficient to alter *Clcn1* RNA splicing, indicating that changes mediated by both denervation and AR113Q toxicity contribute to altered RNA processing. To test this notion directly, we exogenously expressed the AR in vitro and observed hormone-dependent changes in the splicing of pre-mRNAs from a human cardiac troponin T minigene. These effects were notably similar to changes mediated by RNA with expanded CUG tracts, but not CAG tracts, highlighting unanticipated similarities between CAG and CUG repeat diseases. The expanded glutamine AR also altered hormone-dependent splicing of a calcitonin/calcitonin gene-related peptide minigene, suggesting that toxicity of the mutant protein additionally affects RNA processing pathways that are distinct from those regulated by CUGBP1. Our studies demonstrate the occurrence of hormone-dependent alterations in RNA splicing in Kennedy disease models, and they indicate that these changes are mediated by both the cell-autonomous effects of the expanded glutamine AR protein and by alterations in skeletal muscle that are secondary to denervation.

INTRODUCTION

Kennedy disease, one of nine degenerative disorders caused by expanded CAG/polyglutamine tracts (Zoghbi and Orr, 2000), results from a mutation in the androgen receptor (AR) that leads to hormone-dependent protein misfolding (Lieberman and Fischbeck, 2000). Men with Kennedy disease often exhibit early myopathic features, develop progressive proximal muscle weakness, and show both lower motor neuron loss and denervation atrophy in skeletal muscle as the disease progresses (Sperfeld et al., 2002; Katsuno et al., 2006). Studies in transgenic mouse models have demonstrated that disease manifestations precede cell death, indicating that cellular dysfunction contributes to the phenotype (Abel et al., 2001; Chevalier-Larsen et al., 2004). However, as with all of the CAG/polyglutamine diseases, the mechanisms leading to these functional deficits are poorly understood.

We recently generated a mouse model of Kennedy disease using gene targeting to exchange 1340 base pairs (bp) of the mouse *Ar* exon 1 with a human sequence containing 21 or 113 CAG repeats (Albertelli et al., 2006; Yu et al., 2006a). Mice expressing the expanded glutamine AR (AR113Q) develop androgen-dependent neuromuscular and systemic pathology that models Kennedy disease (Yu et al., 2006b; Yu et al., 2006a), whereas AR21Q mice are similar to wild-type littermates (Albertelli et al., 2006). AR113Q skeletal muscle shows both denervation and myopathy, including decreased expression of skeletal muscle chloride channel 1 (CLC-1, encoded by the *Clcn1* gene) (Yu et al., 2006b).

Expression of CLC-1 is also decreased in myotonic dystrophy (DM), a multisystem disorder that features prominent neuromuscular pathology. DM is caused by CUG or CCUG repeat expansions in noncoding regions (Brook et al., 1992; Fu et al., 1992; Liquori et al., 2001) that are pathogenic at the RNA level (Osborne and Thornton, 2006; Ranum and Cooper, 2006). These toxic RNAs cause altered expression of a limited set of RNA-binding proteins, resulting in a disruption of pre-mRNA splicing (Ranum and Day, 2004). This dysregulation of RNA processing affects several transcripts and leads to the aberrant inclusion of exon 7a in *Clcn1* mRNA (Charlet et al., 2002; Mankodi et al., 2002). This exon is normally present in fetal, but not adult, mRNAs and its inclusion leads to nonsense-mediated decay owing to an in-frame stop codon.

Here, we explore whether similar changes in RNA splicing contribute to decreased CLC-1 expression in Kennedy disease mice. We demonstrate altered splicing of *Clcn1* and muscleblind-like protein 1 (*Mbnl1*) RNAs in AR113Q skeletal muscle, which is similar to the defects in DM. These missplicing events are associated with increased expression of CUGBP1, an RNA-binding protein that is implicated in DM pathogenesis. Notably, CUGBP1 expression is similarly increased by surgical denervation of wild-type muscle, however this is not sufficient to lead to changes in *Clcn1* RNA splicing. Our data demonstrate that cell-autonomous mechanisms triggered by AR113Q protein toxicity also contribute to alterations in splicing. We suggest that changes in RNA processing mediated by toxic effects of the expanded glutamine AR contribute to the cellular dysfunction that occurs in this disease.

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RESULTS

Altered RNA splicing and RNA-binding protein expression in AR113Q muscle

Expansion of the CAG/glutamine tract in the AR causes Kennedy disease and triggers androgen-dependent neuromuscular pathology. Among the changes in skeletal muscle from a knock-in mouse model of Kennedy disease is a hormone-dependent, ~25-50% decrease in the expression of mRNA encoding the chloride channel CLC-1 (Yu et al., 2006b). Expression of this channel is also reduced in both DM skeletal muscle and mouse models owing to the aberrant inclusion of exon 7a (Mankodi et al., 2002; Kanadia et al., 2003). This RNA missplicing in DM results in the incorporation of an in-frame stop codon and nonsense-mediated mRNA decay. Because the same chloride channel is expressed at diminished levels in both AR113Q and DM muscle, we examined whether similar RNA missplicing occurs in skeletal muscle from Kennedy disease mice.

We used quantitative real-time PCR (qPCR) to test whether altered pre-mRNA splicing occurs in AR113Q skeletal muscle. The amount of exon 7a-containing transcripts relative to the total pool of *Clcn1* mRNA increased by ~2–3-fold in adult AR113Q males compared with wild-type littermates (Fig. 1A). This effect was observed in both proximal hind limb (left and middle panels) and levator ani/bulbocavernosus (LA/BC) muscles (right panel), the latter of which expresses high levels of AR protein. RNA missplicing was eliminated by surgical castration of young mutant males (left panel), a manipulation that abrogates AR113Q toxicity and increases total *Clcn1* mRNA to near wild-type levels (Yu et al., 2006b). Similar RNA missplicing was detected in AR113Q males that were bred on a more uniform genetic background and compared with AR21Q males (middle panel). We conclude that hormone- and glutamine length-dependent alterations in *Clcn1* pre-mRNA splicing occur in AR113Q mice. We noted that the fold increase in exon 7a inclusion was smaller when comparing AR113Q mice with AR21Q mice on a more uniform genetic background than with wild-type mice on a mixed genetic background. We sought to determine whether this difference was because of an influence of strain background. We directly compared the *Clcn1* mRNA splicing changes in mice with different genetic backgrounds in a single qPCR experiment (supplementary material Fig. S1). No significant difference was detected between N2 and N10 AR113Q males, indicating that the strain background did not influence *Clcn1* mRNA splicing. By contrast, exon 7a inclusion was significantly higher in AR21Q mice than in wild-type mice. This may reflect differences in activity between the humanized 21Q AR and the endogenous mouse receptor.

DM is characterized by the altered expression of several RNA-binding proteins, with the consequence that developmentally regulated splicing events involving multiple transcripts are misregulated and adult splicing patterns are not expressed appropriately (Lin et al., 2006). We next determined whether the missplicing of two other transcripts that are affected in DM occurs similarly in AR113Q muscle. We observed a modest, yet significant, increase of *Mbnl1* RNA missplicing, with the aberrant incorporation of exon 7 into transcripts in LA/BC and proximal hind limb muscle, the latter of which was detected in mice on a uniform genetic background (Fig. 1B, middle and right panels). A similar, hormone-dependent trend toward increased missplicing was observed in AR113Q mice on a mixed genetic background, however these changes did not reach statistical significance (left panel). By contrast, alteration in sarcoplasmic reticulum Ca(2+)-ATPase 1 (*Serca1*, also known as *Atp2a1*) RNA splicing affecting the inclusion of exon 22 did not occur (supplementary material Fig. S2), indicating that only a subset of missplicing events in DM muscle also occurred in AR113Q males.

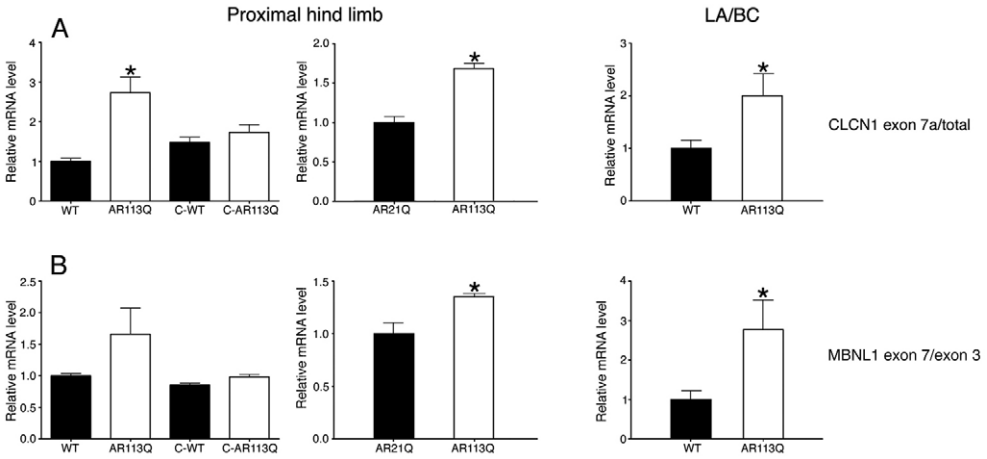

Fig. 1. Hormone- and glutamine length-dependent RNA missplicing in AR113Q muscle. (A,B) Splicing of *Clcn1* and *Mbnl1* RNA was assessed by determining the abundance of transcripts containing exon 7a relative to total *Clcn1* mRNA (A) and exon 7 relative to exon 3-containing *Mbnl1* mRNA (B). RNA expression levels were determined by qPCR in proximal hind limb (left and middle columns) and levator ani/bulbocavernosus muscles (LA/BC, right column) harvested from mice that were castrated at puberty or left intact. Data are reported as mean±S.D. relative to wild type (WT) or AR21Q. The mice evaluated were littermate WT (n=6), AR113Q (n=6), castrated WT (C-WT, n=6) and castrated AR113Q males (C-AR113Q, n=5) on a mixed C57BL/6J-129 genetic background [left and right columns; these mice were backcrossed to C57BL/6J mice for two generations (designated N2)]. The middle column compares AR21Q (n=5) mice that were backcrossed to C57BL/6J mice for eight generations (designated N8) with AR113Q mice (n=3) that were backcrossed to C57BL/6J mice for ten generations (designated N10). *P<0.05.

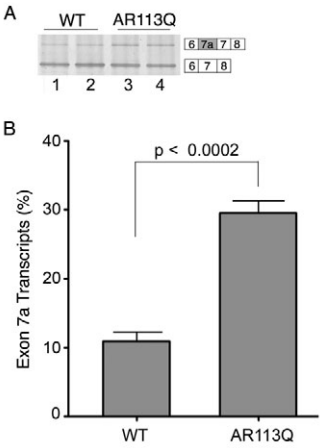

Fig. 2. Increased inclusion of exon 7a in *Clcn1* RNA from AR113Q muscle. (A) Semi-quantitative RT-PCR of *Clcn1* RNA from littermate N2 WT (lanes 1, 2) and AR113Q (lanes 3, 4) proximal hind limb muscle, using primers in exons 6 and 8, demonstrates increased inclusion of exon 7a (top band) in AR113Q muscle. PCR products were resolved on a 15% nondenaturing polyacrylamide gel and stained with SYBR Green. (B) PCR products were stained with SYBR Green and quantified as described in the Methods to determine the percentage of exon 7a-containing *Clcn1* RNA in littermate N2 WT (n=4) and AR113Q (n=4) proximal hind limb muscle. Data are reported as mean±S.D.

To determine the extent of *Clcn1* RNA missplicing in AR113Q muscle, we used a semi-quantitative reverse transcription PCR (RT-PCR) assay to establish the percentage of aberrantly spliced transcripts. Primers that anneal to exons 6 and 8 amplified a major product of 186 bp from cDNAs lacking exon 7a, and a 265-bp product from cDNAs containing exon 7a. Following PCR amplification through an increasing number of cycles, these bands were resolved on nondenaturing polyacrylamide gels, stained by SYBR Green, and quantified to determine the linear range of sensitivity for this assay (supplementary material Fig. S3). Our analyses detected the inclusion of exon 7a in approximately 10% of *Clcn1* transcripts in the skeletal muscle of wild-type mice (Fig. 2A,B), similar to the rate of inclusion that was reported previously (Charlet et al., 2002; Mankodi et al., 2002). Exon 7a inclusion increased significantly to almost 30% of *Clcn1* transcripts in AR113Q muscle (Fig. 2A,B). Notably, some even larger *Clcn1* RNA splice variants, which have been demonstrated in muscle from DM patients and mouse models, were not detected in AR113Q muscle (Charlet et al., 2002; Mankodi et al., 2002).

The alterations of RNA splicing in AR113Q skeletal muscle were associated with increased expression of an RNA-binding protein that has been implicated in DM pathogenesis (Timchenko et al., 1996; Philips et al., 1998). We detected a significant induction of CUG triplet repeat, RNA binding protein 1 (*Cugbp1*) mRNA and protein (Fig. 3A,C) in proximal hind limb and LA/BC muscles. These changes occurred in a hormone- and glutamine length-dependent manner and paralleled the occurrence of altered *Clcn1* RNA splicing. By contrast, AR113Q skeletal muscle showed no change in the overall expression levels of *Mbnl1* mRNA or protein (Fig. 3B,C), which is another RNA-binding protein that has been implicated in DM splicing alterations.

Denervation increases CUGBP1 expression but does not alter *Clcn1* RNA splicing

Next, we sought to determine the extent to which changes in RNA splicing in AR113Q skeletal muscle were the result of non-cell-autonomous effects mediated by loss of innervation by motor neurons. Since androgen-dependent motor neuron degeneration occurs in Kennedy disease, we examined whether changes in RNA splicing and RNA-binding protein expression could be replicated in denervated muscle in the absence of a microsatellite expansion. Wild-type male mice underwent unilateral sciatic nerve transection, and denervated and intact gastrocnemius muscles were harvested after 72 hours. Surgical denervation increased the expression of *MyoD* (also known as *Myod1*) and acetylcholine receptor α-subunit

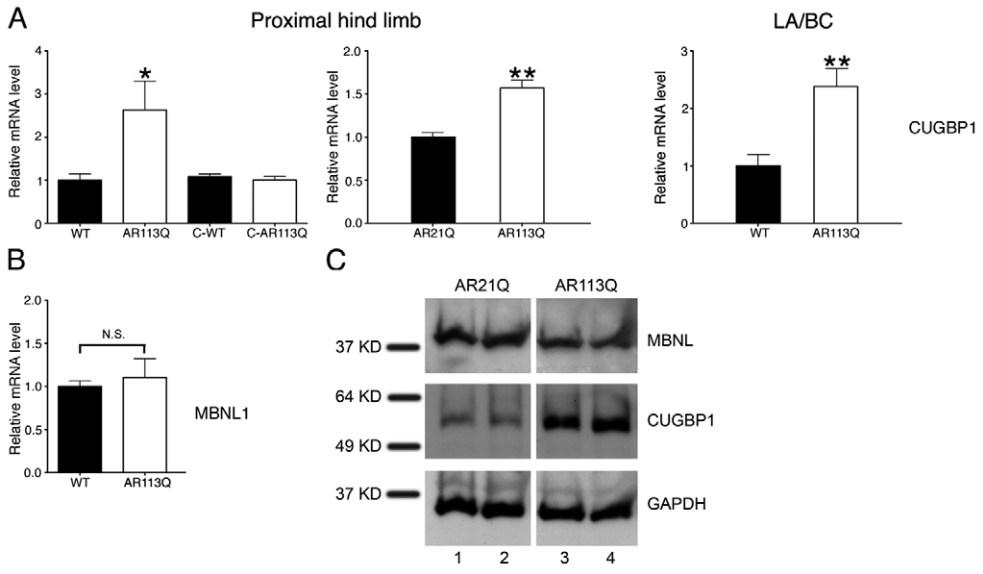

Fig. 3. Increased CUGBP1 expression in AR113Q muscle. (A) *Cugbp1* mRNA levels are reported relative to 18s rRNA. Expression levels were determined by qPCR in proximal hind limb (left, middle columns) and LA/BC (right column) muscles, and are reported as mean±S.D. relative to WT or AR21Q muscles. The mice evaluated were littermate N2 WT (n=6), AR113Q (n=6), castrated WT (C-WT, n=6) and castrated AR113Q males (C-AR113Q, n=5) (left, right columns), and N8 AR21Q (n=5) and N10 AR113Q (n=3) mice (middle column). *P<0.05, **P<0.005. (B) Relative *Mbnl1* mRNA levels in the proximal hind limb muscles of littermate N2 WT (n=6) and AR113Q (n=6) males, reported as mean±S.D. N.S., not significant. (C) MBNL and CUGBP1 expression in the proximal hind limb muscles of N8 AR21Q (lanes 1, 2) and N10 AR113Q (lanes 3, 4) mice was determined by western blot. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) controls were used for loading.

mRNAs, indicating that the expected changes in gene expression were detected in denervated samples (Klocke et al., 1994; Kostrominova et al., 2005; Mejat et al., 2005) (Fig. 4A). Denervated muscle also exhibited significantly increased expression of *Cugbp1* mRNA and protein (Fig. 4B,C), similar to that observed in AR113Q muscle. Despite the complete denervation mediated by sciatic nerve transection and the robust induction of CUGBP1, no significant change in *Clcn1* mRNA splicing was detected (Fig. 4D). We conclude that denervation can mediate some effects on RNA-binding protein expression, but that these are not sufficient to induce the changes in RNA processing that are seen in AR113Q muscle.

Expanded glutamine AR protein affects RNA splicing

Since the changes in muscle following denervation did not trigger the alterations in RNA processing that were observed in AR113Q mice, we reasoned that there must be additional effects arising in skeletal muscle from the expanded CAG *Ar* allele. We first sought to determine whether a toxic RNA could be implicated in this process. Bidirectional transcripts across microsatellite expansions have been detected at the spinocerebellar ataxia 8 (*SCA8*, also known as *ATXN8OS*) and *DM1* loci (Cho et al., 2005; Moseley et al., 2006), and in permutation carriers of the *FMR1* repeat expansion (Ladd et al., 2007), and could generate CUG-containing RNA if they were to occur at *Ar* exon 1. However, nested linker-primer PCR failed to detect specific antisense transcripts in AR113Q muscle (data not shown). Additionally, fluorescence in situ hybridization did not detect intranuclear RNA foci containing either CAG or CUG transcripts in AR113Q muscle, whereas skeletal muscle from a mouse model of DM1 (Mankodi et al., 2000) contained abundant intranuclear CUG foci (data not shown). We also considered the possibility that sense transcripts could alter splicing regulation in AR113Q muscle. However, qPCR demonstrated that the abundance of *Ar* mRNA did not change after surgical castration (data not shown), even though this manipulation eliminated RNA missplicing. Taken together, these studies provided no evidence to support the notion that a toxic *Ar* RNA species that contributes to splicing misregulation is present in AR113Q muscle.

We therefore considered the possibility that the expanded glutamine AR protein exerts effects on RNA splicing. To test this hypothesis, we used a human cardiac troponin T (cTNT) minigene that was previously shown to reproduce the aberrant splicing of endogenous cTNT in DM (Ho et al., 2005b; Singh and Cooper, 2006). This minigene includes exon 5 of cTNT and the flanking intronic sequence containing MBNL1 and CUBBP1 binding sites (Fig. 5A). We initially expressed this minigene and used primers that anneal to sequences flanking exon 5 to generate PCR products from cDNA following an increasing number of amplification cycles. These bands were resolved on nondenaturing polyacrylamide gels, stained by SYBR Green, and quantified to determine the linear range of assay sensitivity (supplementary material Fig. S4). Based on these results, a constant amount of the cTNT minigene was used as a reporter and co-expressed with either AR112Q or a truncated DMPK minigene containing exons 11 through to 15. These DMPK minigenes generate mRNAs containing 960 interrupted repeats of either CUG or CAG in their 3′ untranslated region (UTR) (Ho et al., 2005b). As reported previously, truncated *DMPK* mRNAs containing 960 CUG repeats altered cTNT RNA splicing and increased exon 5 inclusion, whereas *DMPK* mRNAs containing 960 CAG repeats had no effect (Fig. 5B). Expression of either AR24Q or AR112Q triggered a

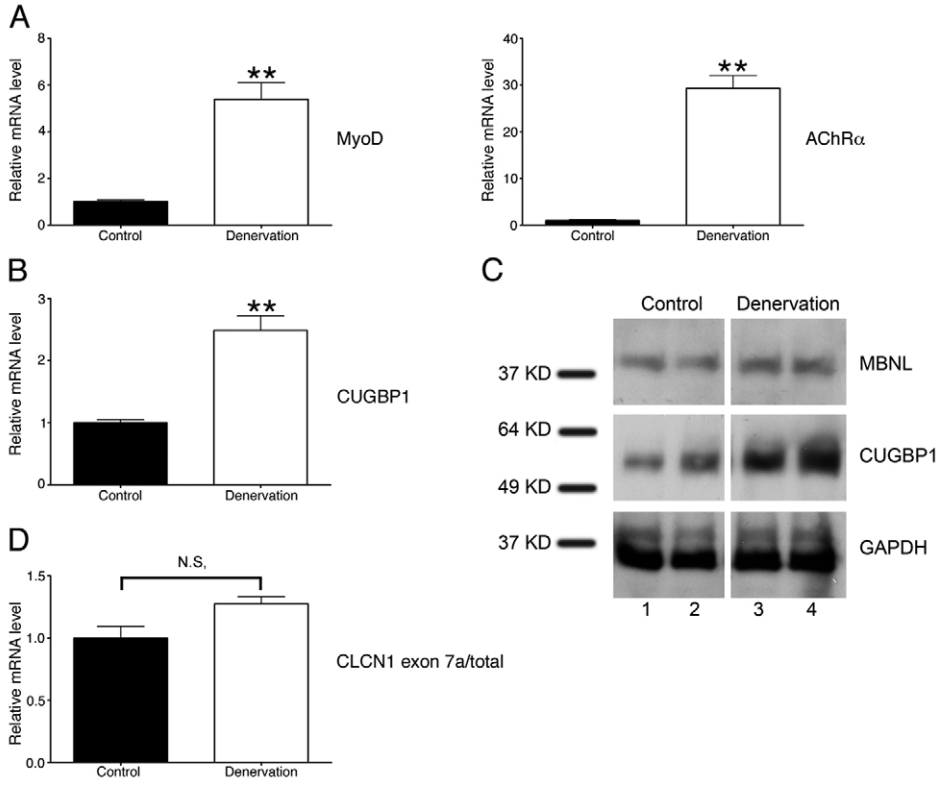

Fig. 4. Increased CUGBP1 expression but no change in *Clcn1* mRNA splicing in denervated muscle. (A) Denervated gastrocnemius muscles and contralateral intact controls were harvested 72 hours after unilateral sciatic nerve transection of wild-type C57BL/6J mice. Relative *MyoD* and acetylcholine receptor α-subunit mRNA expression was determined by qPCR. Data are reported as mean±S.D. relative to 18s rRNA (n=3 per group). $^{**}P<0.005$. (B) Relative *Cugbp1* mRNA levels were determined by qPCR. Data are reported as mean±S.D. relative to 18s rRNA (n=3 per group). $^{**}P<0.005$. (C) MBNL and CUGBP1 expression in control (lanes 1, 2) and denervated (lanes 3, 4) gastrocnemius muscle was determined by western blot. GAPDH was used as a loading control. (D) *Clcn1* RNA splicing was assessed by qPCR by determining the abundance of transcripts containing exon 7a relative to the total *Clcn1* transcripts (n=3 mice per group). N.S., not significant.

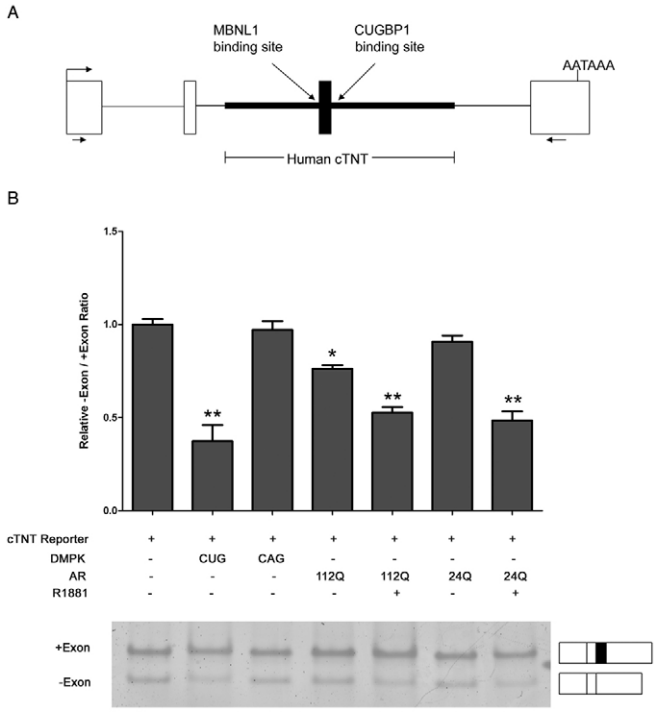

Fig. 5. AR112Q protein alters splicing of cTNT minigene RNA. (A) Schematic of the human cTNT minigene [modified from Singh and Cooper (Singh and Cooper, 2006)] containing exon 5 and the flanking intron sequences (dark lines), including the MBNL1 and CUGBP1 binding sites. The location of the PCR primers is indicated by arrows. (B) Splicing of cTNT minigene RNA in HeLa cells assessed after cotransfection with the pcDNA control plasmid, DMPK minigenes, AR24Q or AR112Q. AR expressing cells were treated with either 10 nM of R1881 (a synthetic androgen) or vehicle for 24 hours. The graph shows relative exon 5 inclusion normalized to cotransfection with pcDNA (mean±S.D.). An image of one of three representative gels is shown below the graph. *P<0.05, **P<0.005 compared with the pcDNA control.

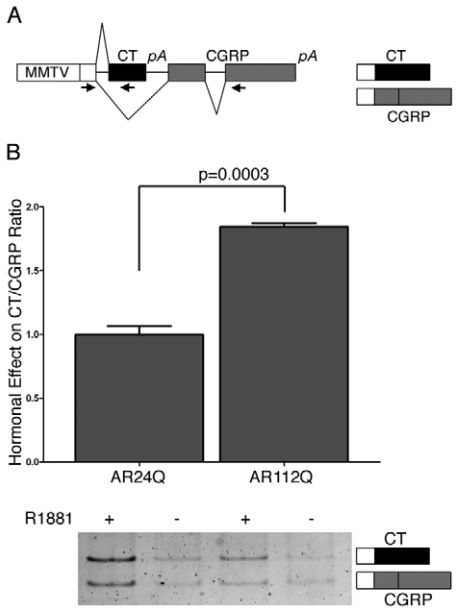

Fig. 6. Ligand-dependent splicing of CT/CGRP minigene RNA is increased by AR glutamine tract length. (A) Schematic of the CT/CGRP minigene [modified from Auboeuf et al. (Auboeuf et al., 2002; Auboeuf et al., 2004)]. The location of the PCR primers is indicated by arrows. (B) Splicing of the CT/CGRP minigene RNA. HeLa cells were cotransfected with the minigene plus either AR24Q or AR112Q, and then treated with 10 nM of R1881 or vehicle for 24 hours. The graph shows the fold hormonal effect on the CT/CGRP ratio (mean±S.D.), relative to cells expressing AR24Q. An image of one of three representative gels is shown below the graph.

ligand-dependent increase of cTNT exon 5 inclusion, similar to that mediated by CUG repeat RNA (Fig. 5B). Since cTNT RNA splicing was not altered by CAG repeat RNA and since the effect of AR was ligand dependent, we conclude that the AR protein alters RNA processing. These observations prompted us to determine whether glutamine length-dependent effects on RNA processing could be identified using a more sensitive system designed to detect the influence of steroid hormone receptors.

Several studies have demonstrated that wild-type forms of steroid hormone receptors (Auboeuf et al., 2002; Auboeuf et al., 2004), including the AR (Dong et al., 2007), interact with the splicing machinery to influence RNA processing. This work raised the possibility that an expanded glutamine tract in the AR could influence RNA splicing more broadly by affecting processing pathways that are not known to be regulated by CUGBP1. To test this possibility, we used a calcitonin/calcitonin gene-related peptide (CT/CGRP) minigene (Fig. 6A). When driven by the hormone-responsive mouse mammary tumor virus (MMTV) promoter, splicing of minigene-derived transcripts is influenced by activation of steroid hormone receptors (Auboeuf et al., 2002; Auboeuf et al., 2004) through processes that couple transcriptional activation with RNA processing (Auboeuf et al., 2007). This CT/CGRP minigene was initially expressed in cells along with AR24Q. Following ligand treatment, PCR primers were used to amplify the CT and CGRP products from cDNA following an increasing number of cycles so that the linear range of assay sensitivity could be established (supplementary material Fig. S5). We then expressed the CT/CGRP minigene with AR24Q or AR112Q and determined the ligand-dependent effects on RNA splicing. Although ligand activation of AR24Q resulted in a modest change in the ratio of CT to CGRP transcripts, ligand activation of AR112Q caused a significantly greater shift in this ratio (Fig. 6B). Quantification of band intensities revealed that hormone activation of the expanded glutamine AR exerted a ~1.8-fold greater effect on RNA splicing than that obtained by activation of the wild-type receptor. These data indicate that the expanded glutamine tract in the AR influences hormone-regulated splicing events and complement previous work that established an effect of the glutamine tract on transcriptional regulation (Mhatre et al., 1993; Chamberlain et al., 1994; Kazemi-Esfarjani et al., 1995; Lieberman et al., 2002).

DISCUSSION

Here, we demonstrate a previously unrecognized consequence of polyglutamine AR toxicity on RNA splicing. AR113Q male mice exhibit hormone- and glutamine length-dependent missplicing of *Clcn1* and *Mbnl1* RNA, and increased expression of the RNA-binding protein CUGBP1. We show that both denervation and cell-autonomous effects in skeletal muscle contribute to these changes

in RNA processing, and that these alterations are the result of the effects of the expanded glutamine AR protein rather than toxic RNA species. Our studies define a novel mechanism augmenting cellular dysfunction in Kennedy disease.

Evidence from cell-based models and mouse models indicate that an altered balance of crucial RNA-binding proteins results in splicing misregulation in DM (Ranum and Cooper, 2006). Two of the RNA-binding proteins implicated in DM pathogenesis, CUGBP1 and MBNL, were initially identified based on their ability to bind to CUG repeat-containing RNA (Timchenko et al., 1996; Miller et al., 2000). Missplicing similar to that which occurs in DM can be reproduced in mice, either by overexpressing CUGBP1 (Ho et al., 2005a) or by diminishing expression of MBNL1 (Kanadia et al., 2003). Studies in an inducible, heart-specific mouse model of DM1 demonstrate that CUGBP1 induction is an early response to the expression of CUG repeat-containing RNA (Wang et al., 2007), and that this effect results from protein kinase C (PKC)-mediated hyperphosphorylation and protein stabilization (Kuyumcu-Martinez et al., 2007). Here, we demonstrate that skeletal muscles from male AR113Q mice similarly exhibit increased expression of CUGBP1 and that this is associated with hormone-dependent changes in *Clcn1* RNA splicing. By contrast, denervation of wild-type muscles induces only CUGBP1 expression and does not lead to alterations in *Clcn1* RNA processing. This observation suggests that other toxic effects of the expanded glutamine AR contribute to splicing changes in Kennedy disease mice.

In addition to the effects on splicing pathways regulated by CUGBP1, our analyses using the MMTV-CT/CGRP minigene indicate that the expanded glutamine AR exerts effects on RNA processing pathways that are influenced by ligand activation of steroid hormone receptors. This notion builds upon data in the literature that establish that the expanded glutamine tract alters transcriptional activity of the AR (Mhatre et al., 1993; Chamberlain et al., 1994; Kazemi-Esfarjani et al., 1995; Lieberman et al., 2002). Much like their interactions with transcriptional co-regulators, steroid hormone receptors including the AR also interact functionally with splicing factors to influence RNA processing (Auboeuf et al., 2002; Auboeuf et al., 2004). The wild-type AR is known to interact in a ligand-dependent manner with PTB-associated splicing factor (PSF) and p54nrb (Dong et al., 2007), which are essential components of the splicing machinery, providing a link between regulators of gene transcription and pre-mRNA splicing. Similarly, the AR coactivator ANT-1 is a U5 small nuclear ribonucleoprotein (snRNP)-associated protein and may recruit U (uridine-rich) snRNPs to transcribed genes (Fan et al., 2006). Our data support a model in which ligand-dependent misfolding, the formation of soluble oligomers, or aberrant interactions between the expanded glutamine AR protein and splicing factors contribute to the misregulation of RNA processing. These findings suggest that alterations in pre-mRNA splicing may result in processing changes that are most manifest in transcripts derived from androgen-regulated genes. This hypothesis is based on the model that transcription and pre-mRNA splicing are coupled events (Orphanides and Reinberg, 2002), and suggests that the effects of the expanded glutamine tract on AR-mediated gene transcription are amplified by alterations in RNA processing.

Our findings provide additional support for the emerging concept of shared mechanisms among repeat expansion diseases that were previously believed to occur through distinct pathways. Bidirectional transcription has been reported at the *DM1, SCA8* and *FMR1* loci, where repeat expansions occur in noncoding regions (Cho et al., 2005; Moseley et al., 2006; Ladd et al., 2007). For *SCA8*, antisense transcripts may result in the production of polyglutamine-containing proteins. Recent studies have also suggested that CAG repeat-containing RNAs are pathogenic in Drosophila (Li et al., 2008). Here, we have demonstrated the occurrence of RNA missplicing and altered RNA-binding protein expression in skeletal muscle from Kennedy disease knock-in mice. These features, associated previously with DM, were unexpected in a model of polyglutamine disease. Our data indicate that the expanded glutamine AR protein, rather than toxic RNA, mediates these effects on RNA processing. This work implicates alterations of RNA splicing as a mechanism through which cell function is affected in Kennedy disease. We suggest that similar changes in RNA processing pathways may contribute to cellular dysfunction in other polyglutamine disorders.

METHODS

Mouse strains

The derivation of mice with targeted *Ar* alleles containing 21 or 113 CAG repeats in exon 1 was described previously (Albertelli et al., 2006; Yu et al., 2006a). Briefly, mice were generated by recombining a portion of human exon 1, encompassing amino acids 31-484, with the mouse *Ar* gene in CJ7 embryonic stem cells. Male chimeras were mated with C57BL/6J females, and females that were heterozygous for the targeted *Ar* allele were bred with wild-type C57BL/6J males to generate the N2 littermate AR113Q and wild-type mice, as well as the N8 AR21Q and N10 AR113Q mice used in this study. Skeletal muscles were harvested from adult male mice at 3-5 months of age with the exception of castrated AR113Q males, which were 18 months of age at the time of muscle procurement. The University of Michigan Committee on Use and Care of Animals approved all procedures involving mice, in accord with the NIH Guidelines for the Care and Use of Experimental Animals.

Orchiectomy

Male mice underwent orchiectomy at 5-6 weeks of age. Following induction of isoflurane anesthesia, the abdomen was cleaned and shaved, and a 1 cm incision was made at the level of the hind legs. The vas deferens and spermatic cord were visualized and ligated with absorbable suture material, the testes removed, and the incision closed with surgical glue.

Sciatic nerve transection

Male C57BL/6 mice (25 g) were used for studies of denervated muscle. Under ketamine-xylazine anesthesia, the right sciatic nerve was exposed at the thigh just below the sciatic notch, ligated with monocryl 4-0 suture, and cut below the ligation to prevent axonal regeneration. Upon recovery, the mice showed evidence of right foot weakness and impaired gait. Seventy-two hours after surgery, the right (operated) and left (control) gastrocnemius muscles were dissected, frozen rapidly in dry ice and processed for RNA isolation.

RNA analysis

Total RNA isolated from tissues with Trizol (Invitrogen, Carlsbad, CA) served as a template for cDNA synthesis using the high capacity cDNA archive kit from Applied Biosystems (Foster City, CA). Gene-

specific primers and probes labeled with a fluorescent reporter dye and quencher were purchased from Applied Biosystems (supplementary material Table S1). TaqMan assays were performed using 5 ng aliquots of cDNA. Replicate tubes were analyzed for the expression of 18s rRNA using a VIC-labeled probe. C_T values were determined by an ABI Prism 7500 sequence detection system, and relative expression levels were calculated using the standard curve method of analysis. Semi-quantitative RT-PCR analysis of *Clcn1* RNA splicing was performed using the 5′ primer 5′-GTCCTCAGC-AAGTTTATGTCC-3′ and the 3′ primer 5′-GAATCCTCGCC-AGTAATTCC-3′ to amplify 20 ng of cDNA through 22 cycles.

Minigenes containing human cTNT exon 5 (RTB300) and DMPK exons 11-15 with interrupted CUG (DT960) or CAG (DM960) repeats were a gift from Dr Thomas Cooper (Baylor College of Medicine). For analysis of reporter gene splicing, HeLa cells grown in 6-well plates were transfected with 1 μg of DNA (500 ng cTNT minigene plus 500 ng pcDNA3, DMPK minigene or AR expression vector) with Fugene 6. Media was removed after 24 hours, and cells were washed and re-fed with Phenol-Red-free DMEM with 10% charcoal-stripped calf serum. Where indicated, cells were treated with 10 nM R1881 or vehicle control. Total RNA was harvested at 48 hours post-transfection with Trizol and cDNA that was prepared using the high capacity cDNA archive kit. Semi-quantitative RT-PCR was performed using the 5′ primer 5′-CATTCACCACATTGGT-GTGC-3′ and the 3′ primer 5′-AGGTGCTGCCGCCGGGCG-GTGGCTG-3′ to amplify 20 ng of cDNA through 20 cycles.

The CT/CGRP minigene driven by the MMTV promoter was a gift from Dr Bert O'Malley (Baylor College of Medicine). For analysis of reporter gene splicing, HeLa cells grown in 6-well plates were transfected with 1 μg of DNA (500 ng CT/CGRP minigene, 50 ng AR24Q or AR112Q, and 450 ng pcDNA3) with Fugene 6. Media was removed after 24 hours, and cells were washed and re-fed with Phenol-Red-free DMEM with 10% charcoal-stripped calf serum supplemented with 10 nM R1881 or vehicle control. Total RNA was harvested at 48 hours post-transfection, as described above. Semi-quantitative RT-PCR was performed using the primers described in Auboeuf et al. (Auboeuf et al., 2002; Auboeuf et al., 2004) to amplify 20 ng of cDNA through 20 cycles.

PCR products (a tenth of the total products) were resolved on 15% nondenaturing polyacrylamide gels and stained with SYBR Green 1 (Invitrogen, Eugene, OR) after electrophoresis. Bands were visualized on a Typhoon Trio+ scanner (Amersham Biosciences, Pistcataway, NJ) and analyzed using AlphaImager 2200 software (Alpha Innotech Corporation, San Leandro, CA).

Protein expression analysis

For western blot analysis, muscle was homogenized in RIPA buffer containing complete protease inhibitor cocktail (Roche, Indianapolis, IN) and 0.1% β-mercaptoethanol using a motor homogenizer (TH115, OMNI International, Marietta, GA). Sample lysates were cleared by centrifugation at 15,000 × ***g*** for 5 minutes at 4°C. Samples were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were probed with antibodies against MBNL1/2/3 (#SC-50088; Santa Cruz Biotechnology, Santa Cruz, CA) and CUGBP1 (#SC-2003; Santa Cruz Biotechnology), and proteins were visualized by chemiluminescence (PerkinElmer Life Sciences, Waltham, MA).

TRANSLATIONAL IMPACT

Clinical issue

Age-associated neurodegeneration is often caused by the accumulation of misfolded or mutant proteins. Many neurodegenerative disorders result from CAG/polyglutamine tract expansions, which produce misfolded proteins that are toxic through mechanisms that are incompletely understood. Nine neurological diseases are known to be caused by expansions of CAG repeats, including Kennedy disease, where a repeat expansion in the androgen receptor (AR) causes hormone-dependent lower motor neuron loss and skeletal muscle pathology.

The authors recently generated a mouse model of Kennedy disease using gene targeting to exchange much of the coding region of the mouse androgen receptor (*Ar*) exon 1 with human sequence containing an expanded CAG repeat that is associated with disease (AR113Q mice). Mice expressing the expanded glutamine AR develop androgen-dependent neuromuscular and systemic disease that models changes in Kennedy disease patients, including skeletal muscle pathology exhibiting evidence of both denervation and myopathy. The mechanism of disease is unknown, and there are no effective treatments available for patients with Kennedy disease or related polyglutamine tract expansion disorders.

Results

Here, using AR113Q mice, the authors identify changes in RNA splicing that decrease the expression of the skeletal muscle chloride channel CLC-1, which is needed to maintain normal membrane potentials in cells. Altered splicing of *Clcn1* RNAs in AR113Q skeletal muscle induces changes similar to those in myotonic dystrophy (DM), a multisystem disorder that is caused by CUG or CCUG repeat expansions in noncoding regions that are pathogenic at the RNA level. RNA missplicing in AR113Q mice also increases the expression of CUGBP1, an RNA-binding protein implicated in DM pathogenesis. CUGBP1 expression is similarly increased by surgical denervation of wild-type muscle but is not sufficient to cause changes in *Clcn1* RNA splicing. Thus, the cell-autonomous mechanisms triggered by AR113Q protein toxicity in muscle result in RNA splicing, which may contribute to the symptoms associated with Kennedy disease and similar neurodegenerative diseases.

Implications and future directions

This work identifies a mechanism augmenting cellular dysfunction in Kennedy disease, whereby the mutant protein alters RNA splicing in a hormone- and glutamine length-dependent manner. The authors suggest that altered RNA splicing may target androgen-regulated genes and amplify the effects of the expanded glutamine tract on AR-mediated gene transcription. These findings support the emerging concept that shared mechanisms exist among repeat expansion diseases, and raise the possibility that changes in RNA processing may contribute to cellular dysfunction in other polyglutamine neurodegenerative disorders.

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Statistics

Statistical significance was assessed by two-tailed Student's *t*-test or by ANOVA with the Newman-Keuls multiple comparison test using the software package Prism 5 (GraphPad Software, San Diego, CA). *P* values less than 0.05 were considered significant.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

A.P.L. and D.M.R. conceived and designed the experiments; Z.Y. and A.M.W. performed the experiments; Z.Y., A.M.W., A.P.L. and D.M.R. analyzed the data; and A.P.L. and D.M.R. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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