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An miRNA-mediated therapy for SCA6 blocks IRES-driven translation of the *CACNA1A* second cistron

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Abstract

Spinocerebellar ataxia type 6 (SCA6) is a dominantly inherited neurodegenerative disease characterized by slowly progressive ataxia and Purkinje cell degeneration. SCA6 is caused by a polyglutamine repeat expansion within a second *CACNA1A* gene product, $\alpha 1$ ACT. $\alpha 1$ ACT expression is under the control of an internal ribosomal entry site (IRES) present within the *CACNA1A* coding region. Whereas SCA6 allele knock-in mice show indistinguishable phenotypes from wild-type littermates, expression of SCA6-associated $\alpha 1$ ACT ($\alpha 1$ ACT_{SCA6}) driven by a Purkinje cell-specific promoter in mice produces slowly progressive ataxia and cerebellar atrophy. We developed an early-onset SCA6 mouse model using an adeno-associated virus (AAV)-based gene delivery system to ectopically express *CACNA1A* IRES-driven $\alpha 1$ ACT_{SCA6} to test the potential of *CACNA1A* IRES-targeting therapies. Mice expressing AAV9-mediated *CACNA1A* IRES-driven $\alpha 1$ ACT_{SCA6} exhibited early-onset ataxia, motor deficits, and Purkinje cell degeneration. We identified miR-3191-5p as a microRNA (miRNA) that targeted *CACNA1A* IRES and preferentially inhibited the *CACNA1A* IRES-driven translation of $\alpha 1$ ACT in an Argonaute 4 (Ago4)-dependent manner. We found that eukaryotic initiation factors (eIFs), eIF4AII and eIF4GII, interacted with the *CACNA1A* IRES to enhance $\alpha 1$ ACT translation. Ago4-bound miR-3191-5p blocked the interaction of eIF4AII and eIF4GII with the *CACNA1A* IRES, attenuating IRES-driven $\alpha 1$ ACT translation. Furthermore, AAV9-mediated delivery of miR-3191-5p protected mice from the ataxia, motor deficits, and Purkinje cell degeneration caused by *CACNA1A* IRES-driven $\alpha 1$ ACT_{SCA6}. We have established proof of principle that viral delivery of an miRNA can rescue a disease phenotype through modulation of cellular IRES activity in a mouse model.

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Data and materials availability: The source data for this study are in the Supplementary Materials. All materials generated are available for distribution.

INTRODUCTION

Spinocerebellar ataxias (SCAs) are a genetically heterogeneous group of dominantly inherited neurodegenerative diseases characterized by progressive ataxia and Purkinje cell degeneration (1–3). To date, more than 30 SCAs have been characterized, each being associated with distinct genes and mutations and therefore requiring individual therapeutic approaches (2, 3). The lack of efficacious therapeutics and the large number of genetic events that result in SCAs have highlighted the need for effective preclinical models to identify and test “druggable” targets.

SCA type 6 (SCA6) is one of the most common forms of autosomal dominant SCAs, representing 10 to 20% of patients with dominantly inherited ataxia. It has an incidence of about 5/100,000 persons (2–8). Patients with SCA6 develop slowly progressive cerebellar ataxia with extensive selective Purkinje cell degeneration, usually beginning at 40 to 50 years of age (2–8). SCA6 is caused by an expanded CAG repeat in the *CACNA1A* gene, which results in an expanded polyglutamine (polyQ) tract. Previous studies unexpectedly found that the expanded polyQ tract does not affect the function or kinetics of the $\alpha 1A$ ($Ca_v2.1$, P/Q-type) voltage-gated Ca^{2+} channel subunit, a gene product of the full-length *CACNA1A* gene (9, 10). Additionally, SCA6 allele knock-in mice were indistinguishable from wild-type littermates, even in old age (9, 10).

We recently discovered that the *CACNA1A* gene is bicistronic, that is, it encodes both the full-length $\alpha 1A$ subunit and a newly recognized transcription factor, $\alpha 1ACT$, consisting of 547 amino acids of the C terminus encoded within a separate open reading frame (ORF) of the same mRNA. The second cistron is translated from a newly identified internal ribosomal entry site (IRES) upstream of the second ORF. We have also characterized the cellular physiological and pathological properties of both wild-type $\alpha 1ACT$ and an expanded polyQ tract containing $\alpha 1ACT$ in vivo, showing that the expanded polyQ tract in $\alpha 1ACT$ results in the SCA6 phenotype. Additionally, we showed that elimination of the portion of the IRES sequence from the human mRNA encoding SCA6-associated $\alpha 1A$ selectively eliminated the expression of the SCA6-associated $\alpha 1ACT$ ($\alpha 1ACT_{SCA6}$) fragment and was protective in a cell culture model of $\alpha 1ACT_{SCA6}$ toxicity (11). Because the complete silencing of *CACNA1A* gene expression would be lethal (11, 12), a more suitable therapeutic approach for SCA6 would be to selectively eliminate expression of $\alpha 1ACT$ while sparing $\alpha 1A$ expression. We reasoned that modulating the expression of microRNAs (miRNAs) targeting the *CACNA1A* IRES could offer a new therapeutic approach for treating SCA6 through regulation of IRES-dependent $\alpha 1ACT_{SCA6}$ translation. On the basis of this hypothesis, we developed a mouse model in which $\alpha 1ACT_{SCA6}$ was expressed in a *CACNA1A* IRES–dependent manner.

RESULTS

Somatic gene transfer of *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ causes Purkinje cell degeneration in mice

We established an in vivo adeno-associated virus type 9 (AAV9) delivery system using the constructs of $\alpha 1\text{ACT}$ transgenes tagged with a C-terminal FLAG epitope, expressed under the control of *CACNA1A* IRES (IRES- $\alpha 1\text{ACT}$ -Q11 and IRES- $\alpha 1\text{ACT}$ -Q33, fig. S1A; AAV9- $\alpha 1\text{ACT}$ -Q11 and AAV9- $\alpha 1\text{ACT}$ -Q33, fig. S1B). We also prepared a control AAV9 vector expressing green fluorescent protein (GFP) (AAV9-GFP). A viral load of 10^{10} vector genomes (vg) of each of these constructs was directly injected into the right lateral ventricle of neonatal wild-type C57/BL6J mice at postnatal day 1. In vivo delivery of AAV9 by intraventricular injection resulted in more than 75% of Purkinje cells being transduced with the AAV9 vector (fig. S1, C and D). Four weeks after AAV9 vector injection, we sacrificed the mice and found widespread expression of $\alpha 1\text{ACT}$ -Q11-FLAG or $\alpha 1\text{ACT}$ -Q33-FLAG that was predominantly observed in the cerebral cortex and cerebellum of wild-type mice injected with AAV9- $\alpha 1\text{ACT}$ -Q11 (AAV9- $\alpha 1\text{ACT}$ -Q11 mice) or AAV9- $\alpha 1\text{ACT}$ -Q33 (AAV9- $\alpha 1\text{ACT}$ -Q33 mice) (Fig. 1, A and B). Quantitative real-time polymerase chain reaction (qRT-PCR) of total RNA from the cerebellum of AAV9-injected mice revealed that $\alpha 1\text{ACT}$ mRNA in the cerebellum of AAV9- $\alpha 1\text{ACT}$ -Q11 and AAV9- $\alpha 1\text{ACT}$ -Q33 mice showed a greater than 2.5-fold up-regulation compared to mouse endogenous *CACNA1A* mRNA in the cerebellum of wild-type mice injected with AAV9-GFP (AAV9-GFP mice). Expression of IRES- $\alpha 1\text{ACT}$ -Q11 mRNA in AAV9- $\alpha 1\text{ACT}$ -Q11 mice was comparable to that of IRES- $\alpha 1\text{ACT}$ -Q33 mRNA in AAV9- $\alpha 1\text{ACT}$ -Q33 mice (Fig. 1C). *CACNA1A* IRES-driven $\alpha 1\text{ACT}$ protein expression in the cerebellum was also comparable between AAV9- $\alpha 1\text{ACT}$ -Q11 and AAV9- $\alpha 1\text{ACT}$ -Q33 mice (Fig. 1D).

We found that, compared with AAV9- $\alpha 1\text{ACT}$ -Q11 mice, AAV9- $\alpha 1\text{ACT}$ -Q33 mice showed a significant decrease in the molecular layer thickness ($P < 0.01$) and the density of the Purkinje cell dendritic tree ($P < 0.01$) (Fig. 1, E to G). We also found that AAV9- $\alpha 1\text{ACT}$ -Q33 caused a 50% loss of Purkinje cells in the cerebellum (Fig. 1H) but no obvious pathological changes in the cerebral cortex and hippocampus (fig. S2, A and B). These pathological features in the cerebellum of AAV9- $\alpha 1\text{ACT}$ -Q33 mice resembled those of Purkinje cell degeneration in patients with SCA6 (13–15).

CACNA1A IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ causes an early-onset ataxia and motor deficits in mice

To assess the behavioral phenotype of AAV9- $\alpha 1\text{ACT}$ -Q33 mice, we used a rotarod performance test, an open-field assay, and a gait stability assessment using a video-assisted computerized treadmill (DigiGait) for gait analysis. AAV9- $\alpha 1\text{ACT}$ -Q33 mice grew at roughly the same rate and had a comparable body weight to AAV9- $\alpha 1\text{ACT}$ -Q11 and AAV9-GFP mice (Fig. 2A). However, AAV9- $\alpha 1\text{ACT}$ -Q33 mice showed defects in motor functions as assessed by the rotarod test (Fig. 2B), activity in the open-field assay (Fig. 2C), and gait stability by the gait analysis assay (Fig. 2, D and E). AAV9- $\alpha 1\text{ACT}$ -Q33 caused impaired performance on the accelerating rotarod test in mice beginning at 4 weeks of age ($P < 0.01$) (Fig. 2B). Ambulatory distance in the open-field test was significantly shorter in AAV9- $\alpha 1\text{ACT}$ -Q33 mice than in AAV9- $\alpha 1\text{ACT}$ -Q11 and AAV9-GFP mice ($P < 0.01$) (Fig. 2C).

DigiGait analyses revealed that AAV9- α 1ACT-Q33 mice exhibited shorter stride length ($P < 0.05$) (Fig. 2D) and greater stride frequencies ($P < 0.05$) (Fig. 2E) in all four limbs from 4 weeks of age, indicating early-onset instability during walking. AAV9- α 1ACT-Q33 mice often showed weaving from side to side on a treadmill, whereas AAV9- α 1ACT-Q11 and AAV9-GFP mice typically walked straight (movies S1 to S3). Furthermore, the deficit in behavioral phenotypes of AAV9- α 1ACT-Q33 mice and continuous *CACNA1A* IRES-driven α 1ACT expression in the cerebellum were observed over a long-term follow-up of 30 weeks (fig. S3, A to G). These findings indicate that AAV9-mediated somatic gene transfer of α 1ACT_{SCA6} under the control of *CACNA1A* IRES caused severe ataxia in mice at much earlier ages and more reproducibly than the Purkinje cell-specific promoter-driven transgenic SCA6 mouse model we previously developed (11).

miR-3191-5p inhibits the *CACNA1A* IRES-driven translation of α 1ACT while sparing α 1A and *CACNA1A* mRNA expression

To develop a *CACNA1A* IRES-directed therapeutic approach for SCA6, we used an miRNA-mediated approach. miRNAs have been increasingly recognized to play a role in the regulation of gene expression, in many cases by both translational repression and mRNA destabilization (16, 17). To date, miRNAs have not been used to preferentially regulate the translation of disease genes driven by a cellular IRES. Although prevailing evidence has pointed to the role of natural miRNAs in gene silencing and translational repression through binding to 3' untranslated regions (UTRs), or rarely 5'UTR, of targeted mRNAs (16–21), predicted miRNA binding sites are found throughout the genome including in both coding and noncoding regions (22, 23).

We used the miRNA_Targets program (22) to predict that miRNAs—miR-711 (accession number, MIMAT0012734), miR-3191-5p (accession number, MIMAT0022732), and miR-4786-3p (accession number, MIMAT0019955)—bind to sequences within the stem-loop structure of *CACNA1A* IRES (fig. S4, A to D) (24). To examine the effects of these miRNAs on *CACNA1A* IRES-driven translation, we cotransfected these miRNAs with either a bicistronic reporter vector bearing *CACNA1A* IRES or a control vector into human embryonic kidney (HEK) 293 cells (Fig. 3A). Dual-luciferase assays revealed that miR-711, miR-3191-5p, and miR-4786-3p down-regulated *CACNA1A* IRES-driven luciferase activities compared to a negative control miRNA (Fig. 3B). We tested the effects of these miRNAs on *CACNA1A*-encoded C-terminal FLAG-tagged peptides (α 1A-FLAG and α 1ACT-FLAG) with the normal (Q11) or pathological (Q33) polyQ tract in transfected HEK293 cells. Although miR-711 and miR-4786-3p decreased full-length α 1A-FLAG and α 1ACT-FLAG expression, miR-3191-5p down-regulated α 1ACT-FLAG expression but spared α 1A-FLAG expression (Fig. 3, C and D). This was the case with α 1A-FLAG and α 1ACT-FLAG in mice with the normal (Q11) or pathological (Q33) polyQ tract. qRT-PCR studies of total RNA showed that miR-711 and miR-4786-3p decreased *CACNA1A* mRNA expression relative to a negative control miRNA, but miR-3191-5p did not affect either *CACNA1A*-Q11 or *CACNA1A*-Q33 mRNA expression (Fig. 3, E and F). We also found that miR-3191-5p did not affect endogenous *CACNA1A* mRNA expression in HEK293 cells (Fig. 3G).

To determine whether miR-3191-5p interacts with regions within the *CACNA1A* mRNA other than the *CACNA1A* IRES, we prepared mutated *CACNA1A* IRES templates, resistant to the binding of miR-3191-5p (*CACNA1A* IRES^{mut}) (fig. S5, A to C). *CACNA1A* IRES^{mut} functioned normally both in the *CACNA1A* IRES^{mut} dual-luciferase assays and in the α 1A-FLAG- and α 1ACT-FLAG-expressing HEK293 cells, but inhibition of expression by miR-3191-5p was prevented (fig. S5, D and E). These results suggest that miR-3191-5p interacts with *CACNA1A* IRES only through its predicted binding site and inhibits the *CACNA1A* IRES-driven translation of α 1ACT while sparing α 1A expression and *CACNA1A* mRNA expression.

Ago4 is required for miR-3191-5p-mediated inhibition of α 1ACT translation

miRNAs generally act on targeted mRNAs in collaboration with the miRNA-induced silencing complex (miRISC)-guided Argonaute (Ago) proteins (25–29). To examine the role of Ago proteins on the inhibitory effects of miR-3191-5p, we cotransfected HEK293 cells with miR-3191-5p and small interfering RNAs (siRNAs) targeting Ago1 to Ago4 carried on either a bicistronic reporter vector bearing the *CACNA1A* IRES or a control vector. Dual-luciferase assays revealed that knockdown of Ago4, but not Ago1 to Ago3, reversed the silencing effects of miR-3191-5p on the *CACNA1A* IRES-driven luciferase activities (Fig. 4A). Western blot analyses showed that silencing of Ago4, but not Ago1 to Ago3, also prevented the down-regulation of α 1ACT-FLAG expression by miR-3191-5p, suggesting that Ago4 is required for miR-3191-5p-mediated inhibition of α 1ACT translation (Fig. 4B).

To determine whether Ago4 directly associates with both miR-3191-5p and *CACNA1A* mRNA or whether Ago4 indirectly mediates the silencing effects of miR-3191-5p, we performed RNA immunoprecipitation-coupled qRT-PCR studies using antibodies specific to Ago1 to Ago4. We found that Ago2- and Ago4-specific antibodies preferentially precipitated miR-3191-5p to a greater extent than did control IgG. Only Ago4-specific antibodies specifically precipitated *CACNA1A* mRNA, indicating that Ago4 selectively bound to both miR-3191-5p and *CACNA1A* mRNA (Fig. 4, C and D). Ago1- and Ago3-specific antibodies had no effect. We also confirmed that knockdown of Ago4 did not affect miR-3191-5p expression in HEK293 cells (Fig. 4E). These results suggest that miR-3191-5p bound to Ago4 directly interacts with the *CACNA1A* IRES and selectively inhibits the *CACNA1A* IRES-driven α 1ACT translation.

miR-3191-5p inhibits the translational initiation of *CACNA1A* IRES-driven α 1ACT by eIF4AII and eIF4GII

Because both eukaryotic initiation factors (eIFs) eIF4A and eIF4G have been previously shown to be involved in IRES-dependent translation in several types of viral IRESs (17), we examined the effects of these on *CACNA1A* IRES-driven α 1ACT translation. We found that overexpression of either eIF4AII or eIF4GII, but not eIF4AI or eIF4GI, increased the *CACNA1A* IRES-driven luciferase activities and that the effects of eIF4AII and eIF4GII were blocked by cotransfection with miR-3191-5p (Fig. 5, A to D). Western blot analyses showed that overexpression of either eIF4AII or eIF4GII increased both α 1A-FLAG and α 1ACT-FLAG expression and that miR-3191-5p reversed the up-regulating effects of eIF4AII and eIF4GII on α 1ACT-FLAG expression without affecting eIF4AII and eIF4GII expression

(Fig. 5, E and F). We also found that overexpression of either eIF4AII or eIF4GII in the presence or absence of miR-3191-5p did not affect *CACNA1A* mRNA expression (Fig. 5, G and H). When we used *CACNA1A* IRES^{mut} templates, the miR-3191-5p-mediated inhibition of the up-regulating effects of eIF4AII and eIF4GII on both *CACNA1A* IRES^{mut} dual-luciferase activities and α 1ACT-FLAG expression were prevented, indicating that the effects of miR-3191-5p depended on its interactions with *CACNA1A* IRES (fig. S6, A to D).

Because both eIF4AII and eIF4GII affected not only *CACNA1A* IRES-driven translation of α 1ACT but also cap-dependent translation of full-length α 1A (Fig. 5, E and F), we used IRES- α 1ACT vectors (fig. S1A) to test the binding affinities of eIF4AII and eIF4GII to *CACNA1A* IRES. RNA immunoprecipitation-coupled qRT-PCR studies revealed that both eIF4AII- and eIF4GII-specific antibodies precipitated IRES- α 1ACT mRNAs to a greater extent than did control IgG, and that binding affinities between IRES- α 1ACT mRNAs and both eIF4AII and eIF4GII were decreased by treatment with miR-3191-5p (Fig. 5, I and J). Coimmunoprecipitation studies revealed that eIF4AII associated with eIF4GII (Fig. 5K). We also found that knockdown of eIF4GII had a greater effect on the down-regulation of *CACNA1A* IRES-driven α 1ACT translation than did knockdown of eIF4AII (Fig. 5L). On the basis of these findings, we concluded that the complex of eIF4AII and eIF4GII directly acts on the *CACNA1A* IRES to enhance IRES-driven α 1ACT translation (fig. S6E). miR-3191-5p bound to Ago4 selectively inhibits the translational initiation of *CACNA1A* IRES-driven α 1ACT by eIF4AII and eIF4GII (fig. S6F).

miR-3191-5p prevents Purkinje cell degeneration in mice caused by *CACNA1A* IRES-driven α 1ACT_{SCA6}

To examine the therapeutic effects of miR-3191-5p-mediated reduction of *CACNA1A* IRES-driven α 1ACT_{SCA6} translation on the disease phenotype of SCA6 mice, we constructed an AAV9 vector that allowed for the simultaneous expression of GFP and either miR-3191-5p (AAV9-miR-3191-5p) or a nonspecific mock miRNA (AAV9-miR-mock) (fig. S7A). We then co-injected AAV9- α 1ACT-Q33 with either AAV9-miR-3191-5p (AAV9-Q33-miR-3191-5p) or AAV9-miR-mock (AAV9-Q33-miR-mock) into the right lateral ventricle of neonatal wild-type C57/BL6J mice at postnatal day 1. Four weeks after AAV9 vector injection, we sacrificed AAV9-injected mice and found widespread transduction of miR-mock and miR-3191-5p throughout the brain and cerebellum (Fig. 6, A and C). We also found a high efficiency (>80%) of co-transduction of AAV9- α 1ACT-Q33 and either AAV9-miR-mock or AAV9-miR-3191-5p in the cerebellum of AAV9-injected mice (fig. S7, B and C). There was a decrease in *CACNA1A* IRES-driven α 1ACT-Q33-FLAG protein expression in the brain and cerebellum of AAV9-Q33-miR-3191-5p mice as compared to AAV9-Q33-miR-mock mice (Fig. 6, B and D). Whereas IRES- α 1ACT-Q33 mRNA expression in the cerebellum of AAV9-Q33-miR-3191-5p mice was comparable to that for AAV9-Q33-miR-mock mice (Fig. 6E), Western blot analyses supported the silencing effect of miR-3191-5p on *CACNA1A* IRES-driven α 1ACT-Q33-FLAG protein expression in the cerebellum of AAV9-Q33-miR-3191-5p mice compared to AAV9-Q33-miR-mock mice (Fig. 6F). Using RNA immunoprecipitation-coupled qRT-PCR studies, we also confirmed that Ago4 bound to both miR-3191-5p and IRES- α 1ACT-Q33 mRNA in the cerebellum of AAV9-Q33-miR-3191-5p mice (Fig. 6, G and H).

Immunohistochemical examination of AAV9-Q33-miR-3191-5p mouse cerebellum showed that the treatment with AAV9-miR-3191-5p protected Purkinje cells from degenerative changes (Fig. 7, A to D). As compared to AAV9-Q33-miR-mock mice, AAV9-miR-3191-5p mice showed protection from thinning of the molecular layer of the cerebellum ($P < 0.01$) (Fig. 7B), decreased density of the dendritic tree ($P < 0.01$) (Fig. 7C), and decreased number of Purkinje cells ($P < 0.01$) (Fig. 7D) caused by AAV9-delivered IRES-driven $\alpha 1\text{ACT-Q33}$. These results indicate that AAV9-delivered miR-3191-5p inhibited Purkinje cell degeneration caused by *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ in mice.

miR-3191-5p prevents the ataxia and motor deficits caused by *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ in mice

We further examined the therapeutic effect of AAV9-miR-3191-5p on mouse behavioral phenotypes caused by *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$. Although the treatment with AAV9-miR-3191-5p did not affect the body weight of AAV9-Q33-miR-3191-5p mice compared to AAV9-Q33-miR-mock mice (Fig. 8A), we found that the *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ -associated disease phenotypes in AAV9-Q33-miR-3191-5p mice were prevented, as assessed by the rotarod test (Fig. 8B), open-field assay (Fig. 8C), and DigiGait for gait analysis (Fig. 8, D and E). AAV9-Q33-miR-3191-5p mice performed significantly better on the accelerating rotarod test ($P < 0.01$) (Fig. 8B) and ambulated a greater distance in the open-field test than did AAV9-Q33-miR-mock mice ($P < 0.01$) (Fig. 8C) at 4 weeks of age. As compared to AAV9-Q33-miR-mock mice, AAV9-Q33-miR-3191-5p mice exhibited improvement of gait instability in all four limbs caused by AAV9-delivered IRES-driven $\alpha 1\text{ACT-Q33}$ ($P < 0.05$) (Fig. 8, D and E). We also found that treatment with AAV9-miR-3191-5p prevented the weaving steps of AAV9-Q33-miR-3191-5p mice (movies S4 and S5). Furthermore, the therapeutic effect of AAV9-miR-3191-5p on mouse behavioral phenotypes and *CACNA1A* IRES-driven $\alpha 1\text{ACT-Q33}$ expression in the cerebellum of AAV9-Q33-miR-3191-5p mice persisted during long-term follow-up of 30 weeks (fig. S8, A to G).

Although both mature and stem-loop sequences of hsa-miR-3191-5p used in our study are identified in the human genome (miRBase: www.mirbase.org/), those of mmu-miR-3191-5p have not yet been identified in the mouse genome. To identify other potential candidates of hsa-miR-3191-5p-targeting mRNAs in mouse, we first used TargetScanHuman 7.0 (www.targetscan.org/vert_70/) to find the top 100 ranked hsa-miR-3191-5p-targeting human mRNAs. We selected 13 genes with high reliability (cumulative weighted context++ score < -1 and total context++ score < -1) (fig. S9A). Among these, seven genes (PRX, ZNF781, C22orf46, ZNF23, ZNF286A, ERBB4, and PTBP1) have hsa-miR-3191-5p binding sites within the 3'UTR of their conserved mouse orthologs. We examined the mRNA levels of these seven genes in the cerebellum of wild-type mice injected with AAV9-miR-3191-5p compared to those injected with AAV9-miR-mock. We found that AAV9-miR-3191-5p down-regulated the mRNA expression of mouse orthologs of ZNF781, C22orf46, ZNF23, and ERBB4 by about 50%, whereas those of the other three genes were unchanged, indicating that not all of the predicted mRNA targets were affected by the treatment with AAV9-miR-3191-5p in mice (fig. S9, B to H).

To determine whether an overall change in whole-gene expression in the mouse brain and cerebellum after AAV9–miR-3191-5p injections leads to significant adverse and toxic effects, we monitored wild-type mice injected with AAV9–miR-3191-5p for 12 months and found no abnormal phenotypes. Also, we found no histopathological abnormalities in the brain, cerebellum, heart, lung, liver, and kidney of wild-type mice injected with AAV9–miR-3191-5p (fig. S10, A to F). On the basis of these findings, we conclude that AAV9-mediated delivery of miR-3191-5p is a well-tolerated and successful therapeutic approach for treating the disease phenotype in our SCA6 mouse model.

DISCUSSION

To explore possible therapeutic strategies for treating SCA6, we developed a robust SCA6 mouse model using AAV9-mediated somatic gene transfer of the *CACNA1A* second cistron to express $\alpha 1\text{ACT}_{\text{SCA6}}$ under the control of *CACNA1A* IRES. We found prominent $\alpha 1\text{ACT}_{\text{SCA6}}$ expression that was predominantly in cerebral cortex and cerebellum of AAV9-injected mice. Mice injected with AAV9 expressing $\alpha 1\text{ACT}_{\text{SCA6}}$ showed unambiguous disease phenotypes associated with the pathological and clinical features of patients with SCA6 (2–8) much earlier and more reproducibly than the Purkinje cell-specific promoter-driven transgenic SCA6 mouse model we previously developed (11) or the knock-in SCA6 mouse model with polyQ expansion size observed in patients with SCA6 (9, 10). By immunofluorescence analysis, we found that administration of a total of 10^{10} vg of AAV9 expressing *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ into the lateral ventricle caused an ~50% decrease in the number of Purkinje cells that was sufficient to lead to early-onset ataxia and motor deficits from an early age. These findings indicate that AAV9-mediated *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ expression is stronger and therefore more toxic in mouse cerebellum than $\alpha 1\text{ACT}_{\text{SCA6}}$ expression in previously developed SCA6 mouse models (9–11). These early and severe ataxic phenotypes are also observed in *Pumilio1*-deficient mice, mimicking SCA1, another type of autosomal dominant SCA (30).

Although we have previously shown that $\alpha 1\text{ACT}$ functions as a transcription factor essential for cerebellar development (11), the detailed mechanism by which $\alpha 1\text{ACT}_{\text{SCA6}}$ causes Purkinje cell degeneration in adulthood remains unknown. Because most individuals with SCA6 are heterozygous for the SCA6-causing expansion, that is, they still have one copy of the normal *CACNA1A* second cistron, they express some levels of the normal $\alpha 1\text{ACT}$ protein. On the other hand, SCA6 homozygotes that express two copies of mutant $\alpha 1\text{ACT}$ protein and no normal $\alpha 1\text{ACT}$ protein develop normally and have nearly the same age of onset as SCA6 heterozygotes (7, 8, 31–33). This confirms that the mutant $\alpha 1\text{ACT}$ protein fulfills some of the normal $\alpha 1\text{ACT}$ protein functions in development and that SCA6 arises by a dominant “gain-of-function” mechanism.

In cell culture experiments, we have discovered that miR-3191-5p directly acts on the *CACNA1A* IRES to regulate the translation of $\alpha 1\text{ACT}$ in an Ago4-dependent manner. Previous studies have shown that miRNAs, by forming miRISC with Ago1 or Ago2, block the assembly of translation initiation factors to repress either cap- or IRES-dependent translation through their binding to the 3' UTR of targeted mRNA (18–20). There are four members of the RNA-induced silencing complex-related Ago protein family in human

Ago1, Ago2, Ago3, and Ago4 (27). Very little is known about Ago4, although the absence of a catalytic domain suggests that it might have a distinct role in gene silencing compared with Ago2 (34–39). Our data showing that Ago4 interacts with miR-3191-5p and *CACNA1A* IRES to repress translation of the second cistron may suggest that it has a specific role for the mRNA-sparing translational repression via miRISC, possibly even selectively for the translational repression of IRESs.

We have also demonstrated that miR-3191-5p bound to Ago4 selectively inhibits the translational initiation of α 1ACT by eIFs, eIF4AII and eIF4GII, which enhance α 1ACT expression. Although the list of cellular mRNAs that are thought to contain IRESs is growing (17, 40–42), cellular IRESs show little structural relationship to each other, and their underlying mechanism remains largely unknown but is thought to follow the picornavirus paradigm of binding to the eIF4A-eIF4G complex (17). Identification of eIF4AII, which may provide helicase activity, and eIF4GII, which may serve as a scaffold (17, 43–45), in the mechanism of *CACNA1A* IRES-mediated translation supports recent findings that show the effect of eIFs on cellular IRESs in human disease (40–42). Presumably, in addition to these eIFs, other IRES transacting factors and small RNAs may play a part in the regulation of *CACNA1A* IRES-mediated translation (17, 40, 42). The recently recognized abundance of IRES-mediated translational mechanisms in the human genome raises the potential for their role in human disease (46).

We have developed an miRNA-mediated therapy for SCA6 using selective translational blockade of the *CACNA1A* second cistron in mice. Our findings indicate that continuous inhibition of α 1ACT_{SCA6} expression by the AAV9 vector-mediated delivery of miR-3191-5p has a substantial therapeutic effect on the SCA6 disease phenotype in mice. Considering that an ~50% decrease in the number of Purkinje cells is sufficient to cause ataxia and motor deficits in our mouse model, miRNA-mediated α 1ACT_{SCA6} silencing by blockade of *CACNA1A* IRES-driven translation in greater than half of Purkinje cells would have great potential benefit in presymptomatic or symptomatic SCA6 patients (13–15).

Although AAV9-mediated intervention with miR-3191-5p did not regulate all of the predicted mRNA targets in our mouse model, we showed that miR-3191-5p has a selective silencing effect on the *CACNA1A* IRES-driven translation of α 1ACT_{SCA6} in mice. Targeting *CACNA1A* IRES in SCA6 patients through miRNAs or small molecules may down-regulate both normal and mutant α 1ACT. However, AAV9-mediated administration of miR-3191-5p into the lateral ventricle did not lead to any toxic effects in mice during long-term observations over 1 year. Other studies in our group also showed that the effect of α 1ACT on establishing normal cerebellar development appeared to be irreversible (47).

There are several limitations to our study. Although patients with SCA6 develop ataxic phenotypes in adulthood (2–8), the targeted expression of α 1ACT_{SCA6} in late adult mouse cerebellum has not been evaluated. Also, the off-target effects due to viral transduction of an miRNA may depend on viral titer and differ among species. A series of reports have suggested that miRNAs may have therapeutic promise for treating cancer, metabolic disease, and inflammation (48–50). However, before miRNAs can have success as therapeutic agents, many challenges remain to be overcome, including their efficient delivery and off-

target effects (51, 52). Further studies are needed to validate more efficient and specific targeting strategies to develop an miRNA-based therapy for SCA6 patients in the future.

Although therapy targeting IRES activity has been a mainstay of antiviral therapy, it has not been used for genetic diseases (53–55). IRES-dependent translation also plays a role in the expression of some oncogenes, such as *c-myc* (56), *L-myc* (57), and epidermal growth factor receptor (58), in tumors. Our study opens the door to the development of therapies using a new strategy for the selective suppression of IRES-driven pathogenic gene products based on delivery of disease-specific miRNAs.

MATERIALS AND METHODS

Study design

Our study was based on our previous findings that SCA6 is attributable to a polyQ repeat expansion within a second *CACNA1A* gene product, $\alpha 1$ ACT, and that $\alpha 1$ ACT expression is under the control of an IRES present within the *CACNA1A* coding region. The objectives of the current study were threefold. The first objective was to develop an early-onset ataxia model using an AAV9-based gene delivery system to express *CACNA1A* IRES-driven $\alpha 1$ ACT_{SCA6}. The second objective was to identify miRNAs that target *CACNA1A* IRES and preferentially block the *CACNA1A* IRES-driven translation of $\alpha 1$ ACT. Finally, we investigated the therapeutic potential of ectopic miRNA expression for the treatment of SCA6 by AAV9-mediated transduction in our previously developed mouse model of *CACNA1A* IRES-driven SCA6. On the basis of our previous studies (11, 59), four to six biological replicates were used for each in vitro or in vivo biochemical and histological analysis, whereas a sample size of 12 mice (6 male and 6 female mice) per group was used for behavioral testing. For AAV9 injections, neonatal mice were randomly assigned to treatment conditions with equivalent numbers in each group. All behavioral analyses were performed by experimenters who were blind to the identity of treatment conditions. Data collection and the biochemical and histological analysis for mouse samples were performed with the investigators unaware of the sample identities until statistical analyses. All source data are in the Supplementary Materials (table S1).

Construction of DNA plasmids

We used pcDNA3 vectors expressing *CACNA1A*-encoded FLAG-tagged peptides ($\alpha 1$ A-FLAG and $\alpha 1$ ACT-FLAG), a bicistronic *CACNA1A* IRES reporter vector, and a control reporter vector as described previously (11). We constructed the truncated transgenes of *CACNA1A* second cistron that lacked the sequence of 5' upstream from *CACNA1A* IRES (pcDNA3-IRES- $\alpha 1$ ACT vectors; fig. S1A) and inserted them into AAV9 genomes to prepare AAV9- $\alpha 1$ ACT-Q11 and AAV9- $\alpha 1$ ACT-Q33 (fig. S1B). Briefly, the sequence of nucleotides 4962 to 7757 of full-length *CACNA1A* complementary DNA (cDNA) (NM_001127222.1), corresponding to the sequence of *CACNA1A* IRES and $\alpha 1$ ACT ORF, was amplified using IRES- $\alpha 1$ ACT primers [5'-ATCAGGATCCGCCCTCAACACCATCGTGC-3' (forward) and 5'-GAATCTAGATTACTTGTCATCG-3' (reverse)] from pcDNA3 vectors expressing *CACNA1A*-encoded FLAG-tagged peptides. The PCR products were inserted into the Bam

HI and Xba I sites of pcDNA3 vectors. We also modified the sequence of nucleotides 5013 to 5015 from “TAT” to “TAG” of stop codon (fig. S1A).

To obtain mutated *CACNA1A* IRES transcripts that are resistant to the binding of miR-3191-5p (*CACNA1A* IRES_{mut} vectors; fig. S5, B and C), we performed C→G and G→C substitutions within the predicted miR-3191-5p binding site. We also performed C→G and G→C substitutions within the complementary binding site of the sequence targeted by miR-3191-5p to maintain the stem-loop structure of *CACNA1A* IRES_{mut} as same as that of the original. Briefly, we amplified the predicted binding site of miR-3191-5p using miR *mut* primers [5′ -

ATGTCTCCGCCCTGGGTCTccccAAcAAcTcTggccCCAGAGTGGCTTACAAGCG-3′ (forward) and 5′ -

CGCTTGTAAGCCACTCTGGggccAgAgTTgTTggggAGACCCAGGGGCGGAGACAT-3′ (reverse)] and the complementary binding site of the sequence targeted by miR-3191-5p using anti-miR *mut* primers [5′ -ACTGAGCA-

CAATAACTTggccAggTTgTTggAGGCCCTCATGCTTCTC-3′ (forward) and 5′ - GAGAAGCATGAGGGCCTccAAcAAcTggccAAGTTATTGTGCTCAGT-3′ (reverse)]. Subsequently, we annealed PCR products using primers anti-miR *mut* forward and miR *mut* reverse and inserted the annealed PCR products into the B1p I sites of pcDNA3 vectors expressing *CACNA1A*-encoded FLAG-tagged peptides and into the Eco RI and Nco I sites of a bicistronic *CACNA1A* IRES reporter vector.

pDEST-GFP-Ago1 (Addgene, plasmid #21534) and pDEST-GFP-Ago4 (Addgene, plasmid #21536) were gifts from E. Chan (60). pEGFP-hAgo2 (Addgene, plasmid #21981) was a gift from P. Sharp (61). pGEX-GST-AGO3 (Addgene, plasmid #24318) was a gift from C. Novina (34). To create pEGFP-AGO3, we restriction-digested pGEX-GST-AGO3 with Eco RI and Bam HI and inserted it into the plasmid backbone of the pEGFP-hAgo2 after Eco RI and Bam HI digestion.

pcDNA3-HA-eIF4GI and pcDNA3-HA-eIF4GII were gifts from N. Sonenberg (McGill University, Canada) (62). M. Bushell (Medical Research Council, UK) provided us with the plasmids expressing human eIF4AI and eIF4AII (18). pcDNA3.1-HisXpress vector was a gift from T. Cooper (Baylor College of Medicine). From the plasmids expressing human eIF4AI and eIF4AII, we amplified human eIF4AI and eIF4AII cDNAs using eIF4AI-PCR primers [5′ -ATCAGGATCCATGTCTGCGAGCCAGGAT-3′ (forward) and 5′ -ATCAGGGCCCAGGTCAGCAACATTGAGG-3′ (reverse)] and eIF4AII-PCR primers [5′ -ATCAGGATCCATGTCTGGTGGCTCCGCG-3′ (forward) and 5′ -ATCAGGGCCCTTAAATAAGGTCAGCCAC-3′ (reverse)], respectively. Subsequently, we inserted the PCR products into Bam HI- and Apa I-digested pcDNA3.1-HisXpress vectors to obtain pcDNA3.1-HisXpress-eIF4AI and pcDNA3.1-HisXpress-eIF4AII. We used the original, either pcDNA3.1-HisC or pcDNA3-HA, vectors lacking the insertions in the multiple cloning sites as control vectors.

Cotransfection of DNA plasmids with either synthetic miRNA or siRNA into cultured cells

HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All miRNAs and siRNAs were purchased from Life Technologies.

ID numbers are shown as follows: miR-711 (MC15715), miR-3191-5p (MC23769), miR-4786-3p (MC21295), a negative control miRNA (#4464059), Ago1 (s25500), Ago2 (s25931), Ago3 (s46947), Ago4 (s46949), eIF4AII (s4570), eIF4GII (s16519), and an siRNA negative control (#12935-300).

We plated HEK293 cells onto six-well dishes and cotransfected each dish with 1.0 µg of the vector expressing the following: bicistronic *CACNA1A* IRES reporter, bicistronic *CACNA1A* IRES^{mut} reporter, control reporter, and α1A-FLAG and α1ACT-FLAG; 0.5 µg of the vector expressing the following: eIF4AI, eIF4AII, eIF4GI, and eIF4GII; and either 20 nM synthetic miRNA or 20 nM siRNA molecules. We used Lipofectamine 2000 (Life Technologies) as a transfection reagent in all cases. Neither the miRNA negative control nor the siRNA negative control matched any human mRNA. The transfected cells were cultured for 48 hours before being processed for RNA and protein analysis.

Luciferase assay

HEK293 cells were plated onto six-well dishes and cotransfected with vectors as shown above. Forty-eight hours after cotransfection, the activities of firefly and *Renilla* luciferase in lysates prepared from transfected cells using the Dual-Luciferase Assay System (Promega) were measured by using a Wallac 1420 VICTOR³ V luminometer with a 1-s integration time (PerkinElmer). The ratio of firefly luciferase to *Renilla* luciferase activities of a bicistronic *CACNA1A* IRES reporter vector was normalized to that of a bicistronic control reporter vector.

Protein expression analysis

We did Western blot analysis as previously described (11, 63). We used the following primary antibodies: FLAG-specific antibody (1:5000, A8592 and 1:5000, F1804; Sigma-Aldrich); Ago1-specific antibody (1:2000, 9388S; Cell Signaling Technology); Ago2-specific antibody (1:2000, SAB4200085; Sigma-Aldrich); Ago3-specific antibody (1:2000, 5054S; Cell Signaling Technology); Ago4-specific antibody (1:2000, 6913S; Cell Signaling Technology); eIF4AII-specific antibody (1:2000, ab31218; Abcam); eIF4GII-specific antibody (1:1000, sc-100732; Santa Cruz Biotechnology); GFP-specific antibody (1:2000, M048-3; MBL); and GAPDH-specific antibody (1:5000, AM4300; Life Technologies). We used NIH ImageJ software to quantify the specific expression of individual proteins and demonstrated the relative signal intensities of individual proteins normalized to those of GAPDH in each sample.

Quantitative real-time polymerase chain reaction

The total RNA was extracted from HEK293 cells and the cerebellum of mice using the miRNeasy Mini Kit (Qiagen) and reverse-transcribed using the SuperScript VILO (Life Technologies) for mRNA and NCode VILO (Life Technologies) for miRNA. The cDNAs were then used for real-time PCR using the iQ SYBR Green Supermix (Bio-Rad Laboratories). We did the amplification, detection, and data analysis using a Bio-Rad iCycler system (Bio-Rad Laboratories). The crossing threshold values for the mRNAs of the individual genes were normalized to β-actin. The crossing threshold values for miR-3191-5p were normalized to U6 small nuclear RNA. Changes in the expression of mRNA and

miRNA were expressed as a fold change relative to the control. We used the following primers. The sequences of the hsa-*CACNA1A* primers were 5'-GTCTGGGGAAGAAGTGTCCTG-3' (forward) and 5'-GCTCCTCCCTTGGCAATCTT-3' (reverse). These hsa-*CACNA1A* primers discriminated between the human *CACNA1A* mRNA and the mouse *CACNA1A* mRNA. The sequences of the hsa-IRES- α 1ACT primers were 5'-GTACCTCACCCGAGACTCCT-3' (forward) and 5'-CGGACACTTCTTCCCCAGAC-3' (reverse). These primers can also detect the mouse endogenous *CACNA1A* mRNA. The sequences of the hsa- β -actin primers were 5'-GCGGGAAATCGTGCGTGACATT-3' (forward) and 5'-GATGGAGTTGAAGGTAGTTTCGTG-3' (reverse). The sequences of the mmu-Prx primers were 5'-TTGGTGGAGATTATCGTGGAG-3' (forward) and 5'-TCTTGCAAGCTGAGGCTCTTA-3' (reverse). The sequences of the mmu-Zfp781 primers were 5'-CCTATGAGGATGTGCATGTGA-3' (forward) and 5'-TGGGGTCCAGAGTGACAGATA-3' (reverse). The sequences of the mmu-4930407I10Rik primers were 5'-GAATCCCAAGGGCTAAAGTG-3' (forward) and 5'-TTTCACACCATCTCCACTTCC-3' (reverse). The sequences of the mmu-Zfp612 primers were 5'-AAGGCAGCCCTCAAGTTAATC-3' (forward) and 5'-AAGTCTCTGATGCCAGACGAA-3' (reverse). The sequences of the mmu-Zfp286 primers were 5'-CATGGAAACCAGACCTGAGAG-3' (forward) and 5'-ACGCTCACATTCAAGAGCAGT-3' (reverse). The sequences of the mmu-ErbB4 primers were 5'-CGCTAGAACTCCACTGATTGC-3' (forward) and 5'-TACCAGCTCTGTCTCCAGGAA-3' (reverse). The sequences of the mmu-Ptbp1 primers were 5'-TCACCAAGAACAACCAGTTCC-3' (forward) and 5'-GTGAGCTTGGAGAAGTCGATG-3' (reverse). The sequences of the mmu- β -actin primers were 5'-GCTACAGCTTCACCACCACA-3' (forward) and 5'-TCTCCAGGGAGGAAGAGGAT-3' (reverse). The sequences of the miR-3191-5p primers were 5'-GCTCTCTGGCCGTCTAC-3' (forward) and 5'-GTCCAGTTTTTTTTTTTTTTTGGGAAG-3' (reverse). The sequences of the U6 small nuclear RNA primers were 5'-CTTCGGCAGCACATATACTAAA-3' (forward) and 5'-AAAATATGGAACGCTTCACG-3' (reverse). We designed the miR-3191-5p primers using a bioinformatics program (64).

Coimmunoprecipitation

We plated HEK293 cells onto 100-mm dishes and cotransfected each dish with 3.0 μ g of the vectors expressing eIF4AII and eIF4GII. Forty-eight hours after transfection, we harvested HEK293 cells for immunoprecipitation using eIF4AII-specific antibody (5 μ g per sample, ab31218; Abcam), eIF4GII-specific antibody (5 μ g per sample, sc-100732; Santa Cruz Biotechnology), and Dynabeads Protein G Immunoprecipitation Kit (Life Technologies) according to the manufacturer's suggested protocols. A rabbit IgG (5 μ g per sample, PP64B; Millipore) and mouse IgG (5 μ g per sample, CS200621; Millipore) were used as controls.

Immunoprecipitation-coupled qRT-PCR

We plated HEK293 cells onto 100-mm dishes and cotransfected each dish with 2.0 μ g of the vector expressing the following: full-length *CACNA1A*-Q33-encoded FLAG-tagged peptides, IRES- α 1ACT-Q33, miR-3191-5p (SC401396; OriGene), Ago1, Ago2, Ago3,

Ago4, eIF4AII, and eIF4GII. Forty-eight hours after transfection, we harvested HEK293 cells for coimmunoprecipitation using Ago1-specific antibody (5 µg per sample, 9388S; Cell Signaling Technology), Ago2-specific antibody (5 µg per sample, 2897S; Cell Signaling Technology), Ago3-specific antibody (5 µg per sample, 5054S; Cell Signaling Technology), Ago4-specific antibody (5 µg per sample, 6913S; Cell Signaling Technology), eIF4AII-specific antibody (5 µg per sample, ab31218; Abcam), eIF4GII-specific antibody (5 µg per sample, sc-100732; Santa Cruz Biotechnology), and Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's suggested protocols. A rabbit IgG (5 µg per sample, PP64B; Millipore) and mouse IgG (5 µg per sample, CS200621; Millipore) supplied by the manufacturer were used as controls. The immunoprecipitated RNA was reverse-transcribed using SuperScript VILO (Life Technologies) for mRNA and NCode VILO (Life Technologies) for miRNA and analyzed by qRT-PCR for the differential expression of *CACNA1A*-Q33 mRNA and IRES- α 1ACT-Q33 mRNA using the following primers: 5'-GTCTGGGGAAGAAGTGTCCG-3' (forward) and 5'-GCTCCTCCCTTGGCAATCTT-3' (reverse), and miR-3191-5p, 5'-GCTCTCTGGCCGTCTAC-3' (forward) and 5'-GTCCAGTTTTTTTTTTTTTTTGAAG-3' (reverse). We also extracted RNA and protein complex from the cerebellum of AAV9-injected mice and harvested them for coimmunoprecipitation as same as shown above.

Development of the AAV9 vectors

The AAV9 vector plasmids contained an expression cassette, consisting of a human cytomegalovirus immediate-early promoter followed by cDNA encoding gene of our interest as shown below, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and a simian virus 40 polyadenylation signal sequence between the inverted terminal repeats of the AAV3 genome. The AAV9 vectors expressing α 1ACT with either normal CAG repeat size (AAV9- α 1ACT-Q11) or mutant CAG repeat size (AAV9- α 1ACT-Q33) contained the truncated transgenes of *CACNA1A* corresponding to the sequence of *CACNA1A* IRES and α 1ACT ORF (IRES- α 1ACT; fig. S1, A and B). AAV9-GFP contained cDNA encoding GFP sequence. AAV9-miR-3191-5p contained cDNA encoding GFP and miR-3191-5p sequence (fig. S7A). AAV9-miR-mock contained cDNA encoding GFP and the miR-mock sequence (fig. S7A). The sequences of miR-3191-5p and miR-mock are 5'-GGGGTCACCTCTCTGGCCGTCTACCTTCCACACTGACAAGGGCCGTGGGGACGTA GCTGGCCAGACAGGTGACCCC-3' (miR-3191-5p) and 5'-GTATTGCGTCTGTACACTCACC GTTTTGGCCACTGACTGACGGTGAGTGCAGACG CAATA-3' (miR-mock).

We synthesized the AAV9 *vp* cDNA as previously described with the substitution of thymidine for adenine 1337, which introduced an amino acid change from tyrosine to phenylalanine at position 446 (65). Recombinant AAV9 vectors were produced by transient transfection into HEK293 cells using the vector plasmid, an AAV3 *rep* and AAV9 *vp* expression plasmid, and the adenoviral helper plasmid pHelper (Agilent Technologies). We purified the recombinant viruses by isolation from two sequential continuous CsCl gradients, and the viral titers were determined by qRT-PCR as follows: 40 cycles of 95°C/15 s, 60°C/30 s, 72°C/90 s, and 75°C/15 s with WPRE forward primer (5'-

ATTGCTTCCCGTATGGCTTTCA-3') and WPRE reverse primer (5'-TCAGCAAACACAGTGCACACCA-3') to amplify the sequence of nucleotides 1319 to 1201 of woodchuck hepatitis virus 2.

Injection of AAV9 into the ventricle of neonatal wild-type mice

The C57/BL6J mice were purchased from Jackson Laboratory and maintained in our breeding colony. At postnatal day 1, neonatal C57/BL6J mice were individually anesthetized on ice, and a total of 10^{10} vg in 2 to 4 μ l of AAV9 solution were injected into the right lateral ventricle of neonatal C57/BL6J mice with a 10- μ l Hamilton syringe attached to a 32-gauge needle (Hamilton Company). The viral solution contained 0.04% trypan blue (Sigma-Aldrich) to help determine whether the ventricles were indeed injected. Only those neonatal C57/BL6J mice in which the lateral ventricles were filled with viral solution were analyzed. Six male and six female mice were enrolled into each group: two groups of AAV9-GFP mice, AAV9- α 1ACT-Q11 mice, AAV9- α 1ACT-Q33 mice, AAV9-Q33-miR-mock mice, and AAV9-Q33-miR-3191-5p mice. All animal experiments were approved and carried out in accordance with the regulations and guidelines for the care and use of experimental animals at the Institutional Animal Care and Use Committee of the University of Chicago.

The behavioral assessments of AAV9-injected mice

We examined the behavioral assessments of AAV9-injected mice at 4, 8, 12, and 30 weeks of age. The investigators who carried out the behavioral assessments were blinded to the treatment conditions.

Rotarod—We analyzed rotarod test of mice using Economex Rotarod (Columbus Instruments) with accelerating mode (4 to 40 rpm, acceleration with 0.1 rpm per 0.8 s). We performed three consecutive trials with 10-min intervals between each trial.

Open-field assay—We examined open-field assay using Mouse Open Field Arena and 48 Channel IR Controller for Open Field Activity (ENV-510 and ENV-520, Med Associates Inc.). Briefly, mice were placed in the center of the open-field area, and their movements were monitored through the side-mounted photobeams for 30 min. We analyzed multiple parameters using Activity Monitor software (Med Associates Inc.) and adopted total distance traveled to assess the activity of each mouse.

DigiGait analysis—We also examined a video-assisted computerized treadmill for mouse gait analysis using a DigiGait with DigiGait software (Mouse Specifics). All mice were tested at the speed of 25 cm/s.

Immunohistochemistry, immunofluorescence, and histopathology

Immunofluorescence was performed as previously described (11, 59) except as modified below. We cut paraffin-embedded sagittally oriented 5- μ m sections of mouse brains and cerebellums at 20- μ m intervals. We used comparable sections from vermis, medial hemisphere, and lateral hemisphere for staining and histopathological assessments. Paraffin-embedded sections of perfused brains and cerebellums were dewaxed, rehydrated, and then steamed for 20 min in antigen retrieval solution (Reveal, Biocare Medical). Sections were

blocked and exposed to primary antibodies for 12 hours at 4°C. After washing, fluorescent secondary antibodies in phosphate-buffered saline and 0.05% Tween 20 were added for 1 hour at room temperature. Confocal fluorescence images were captured with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems Inc.).

We used NIH ImageJ software to quantify the FLAG and GFP expression in each section and demonstrated the relative signal intensities of the FLAG and GFP fluorescence per unit area of the mouse hippocampus, cerebral cortex, and cerebellum in each section. The molecular layer thickness and density of Purkinje dendritic trees were calculated as previously described (11, 66). We selected Purkinje cells well stained with GFP- and FLAG-specific antibodies, indicating well transduced with AAV9, and analyzed Purkinje cells (100 to 250 cells per sample) in the entire area of each section to calculate the mean of the density of Purkinje dendritic trees. The dendritic trees of the captured Purkinje cell image and the area enclosed were outlined and measured using NIH ImageJ software. We also calculated the number of Purkinje cells (100 to 250 cells per sample) in the entire area of each section and expressed the results as the number per 250 μm .

We used the following primary antibodies: FLAG-specific antibody (1:200, F1804 and 1:200, F7425; Sigma-Aldrich), GFP-specific antibody (1:200, M048-3; MBL), and calbindin-specific antibody (1:200, CB38a; Swant). Goat Alexa Fluor-conjugated anti-mouse and goat Alexa Fluor-conjugated anti-rabbit IgG antibodies (Life Technologies) were used for secondary fluorescence detection. For the tissue sections stained with hematoxylin and eosin, digital image files were created with a 3D Histech Panoramic Scan whole slide scanner (PerkinElmer) with a Stingray F146C color camera (Allied Vision Technologies). Individual images were analyzed using the 3D Histech Panoramic Viewer software (PerkinElmer).

Statistical analysis

Statistical analysis was performed using ANOVA and Student's *t* test, unless specified, with the IBM SPSS Statistics 23.0. Two-tailed unpaired *t* test was used to compare two conditions. One-way ANOVA was used for comparison among multiple experimental conditions. Bonferroni post hoc test was used when comparing among each condition. For the analysis of mouse body weight and rotarod performance, two-way ANOVA was used for comparison among groups. All data represent means $\pm$ SEM. Statistical significance in figures: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Somatic gene transfer of *CACNA1A* IRES-driven $\alpha 1$ ACT_{SCA6} causes Purkinje cell degeneration in mice

(A) Representative immunofluorescence images of AAV9-injected mouse brain and cerebellum from 4-week-old mice stained with FLAG-specific antibodies and fluorescent secondary antibody. Scale bar, 5 mm. (B) Relative $\alpha 1$ ACT-FLAG immunofluorescence intensities in AAV9-injected mouse hippocampus, cerebral cortex, and cerebellum ($n = 6$). Relative $\alpha 1$ ACT-FLAG immunofluorescence is expressed as signal intensities per unit area quantified by the National Institutes of Health (NIH) ImageJ software. n.s., not significant. (C) Relative $\alpha 1$ ACT mRNA expression in the cerebellum of AAV9-injected mice at 4 weeks of age ($n = 4$). (D) Western blot and densitometric analyses showing $\alpha 1$ ACT-Q11-FLAG and $\alpha 1$ ACT-Q33-FLAG expression in the cerebellum of AAV9-injected mice at 4 weeks of age ($n = 4$). (E) Representative immunofluorescence images of AAV9-injected mouse cerebellum from 4-week-old mice stained with calbindin-specific antibodies and fluorescent secondary antibody. Scale bar, 100 μ m. (F to H) Molecular layer thickness (F), density of dendritic tree (G), and Purkinje cell count of AAV9-injected mouse cerebellum from 4-week-old mice ($n = 6$) (H). GFP, AAV9-GFP mice; Q11, AAV9- $\alpha 1$ ACT-Q11 mice; Q33, AAV9- $\alpha 1$ ACT-Q33 mice. All data represent means $\pm$ SEM. ** $P < 0.01$. Student's t test in (B) to (D). One-way analysis of variance (ANOVA) in (F) to (H).

Fig. 2. *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ causes an early-onset ataxia and motor deficits in mice

(A to E) Clinical features of AAV9-injected mice. Body weight (A), rotarod test (B), and open-field assay (C) at 4 weeks of age. Stride length (D) and stride frequencies (E) assessed by DigiGait analysis at 4 weeks of age ($n = 12$, 6 male and 6 female mice per group). GFP, AAV9-GFP mice; Q11, AAV9- $\alpha 1\text{ACT}$ -Q11 mice; Q33, AAV9- $\alpha 1\text{ACT}$ -Q33 mice. All data represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. Two-way ANOVA in (A) and (B). One-way ANOVA in (C) to (E).

Fig. 3. miR-3191-5p inhibits the *CACNA1A* IRES-driven translation of α 1ACT while sparing α 1A and *CACNA1A* mRNA expression in HEK293 cells

(A) Schematic representation of a bicistronic control reporter vector and a bicistronic *CACNA1A* IRES reporter vector. SV40, simian virus 40; polyA, polyadenylation signal sequence. (B) Relative dual-luciferase *CACNA1A* IRES reporter activities in HEK293 cells treated with miR-711, miR-3191-5p, miR-4786-3p, or an miRNA negative control (NC) ($n = 6$). The ratio of firefly luciferase to *Renilla* luciferase activities of a bicistronic *CACNA1A* IRES reporter vector was normalized to that of a bicistronic control reporter vector. (C and D) Western blot and densitometric analyses showing α 1A-FLAG and α 1ACT-FLAG expression in HEK293 cells treated with three miRNAs or NC ($n = 6$). (C) *CACNA1A*-Q11. (D) *CACNA1A*-Q33. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (E and F) Relative *CACNA1A*-Q11 (E) and *CACNA1A*-Q33 (F) mRNA expression in HEK293 cells treated with three miRNAs or NC ($n = 6$). (G) Relative endogenous *CACNA1A* mRNA expression in HEK293 cells treated with miR-3191-5p or NC ($n = 6$). All data represent means $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$. Student's t test in (B) to (G).

Fig. 4. Ago4 is required for miR-3191-5p-mediated inhibition of *CACNA1A* IRES-driven α 1ACT translation

(A) Relative dual-luciferase *CACNA1A* IRES reporter activities in HEK293 cells treated with si-Ago1, si-Ago2, si-Ago3, si-Ago4, or a scrambled control siRNA (si-C) in the presence of miR-3191-5p or an miRNA NC ($n = 6$). The ratio of firefly luciferase to *Renilla* luciferase activities of a bicistronic *CACNA1A* IRES reporter vector was normalized to that of a bicistronic control reporter vector. (B) Western blot and densitometric analyses showing α 1A-Q33-FLAG and α 1ACT-Q33-FLAG expression in HEK293 cells treated with four siRNAs or si-C in the presence of miR-3191-5p or NC ($n = 6$). (C and D) Amount of miR-3191-5p (C) and *CACNA1A*-Q33 (D) mRNA binding to Ago1 to Ago4 that was immunoprecipitated using antibodies against Ago1 to Ago4 or a control immunoglobulin G (IgG) ($n = 6$). (E) Relative amount of endogenous miR-3191-5p in HEK293 cells treated with si-Ago4 or si-C ($n = 6$). All data represent means $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$. Student's t test in (A) to (E).

Fig. 5. miR-3191-5p inhibits the translational initiation of *CACNA1A* IRES-driven α 1ACT by eIF4AII and eIF4GII

(A to D) Relative dual-luciferase *CACNA1A* IRES reporter activities in HEK293 cells treated by overexpression of either eIF4AI (AI) (A), eIF4AII (AII) (B), eIF4GI (GI) (C), eIF4GII (GII) (D), or a control vector (C) in the presence of miR-3191-5p or an miRNA NC ($n = 6$). The ratio of firefly luciferase to *Renilla* luciferase activities of a bicistronic *CACNA1A* IRES reporter vector was normalized to that of a bicistronic control reporter vector. (E and F) Western blot and densitometric analyses showing α 1A-Q33-FLAG and α 1ACT-Q33-FLAG expression in HEK293 cells treated by overexpression of either AII (E), GII (F), or C in the presence of miR-3191-5p or NC ($n = 6$). (G and H) Relative *CACNA1A*-Q33 mRNA expression in HEK293 cells treated by overexpression of either AII (G), GII (H), or C in the presence of miR-3191-5p or NC ($n = 6$). (I and J) Amount of IRES- α 1ACT-Q33 mRNA binding to AII (I), GII (J), or a control IgG in the presence of vectors

expressing miR-3191-5p (miR-3191) or an miRNA control (miR-C) that was immunoprecipitated using antibodies against AII, GII, or a control IgG ($n = 6$). (K) Immunoprecipitation (IP) from HEK293 cells transfected with AII- and GII-expressing vectors using an antibody against AII, GII, or a control IgG. Recovered proteins were analyzed using Western blot. Input, 10%. (L) Relative dual-luciferase *CACNA1A* IRES reporter activities in HEK293 cells treated with si-AII, si-GII, or a scrambled si-C ($n = 6$). The ratio of firefly luciferase to *Renilla* luciferase activities of a bicistronic *CACNA1A* IRES reporter vector was normalized to that of a bicistronic control reporter vector. All data represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Student's t test in (A) to (F), (I), (J), and (L). One-way ANOVA in (G) and (H).

Fig. 6. AAV9-mediated therapeutic delivery of miR-3191-5p blocks *CACNA1A* IRES-driven $\alpha 1$ ACT translation in mice

(**A** and **B**) Representative immunofluorescence images of AAV9-injected mouse brain and cerebellum from 4-week-old mice stained with GFP-specific (**A**) and FLAG-specific (**B**) antibodies and fluorescent secondary antibody. Scale bar, 5 mm. (**C** and **D**) Relative GFP (**C**) and $\alpha 1$ ACT-FLAG (**D**) immunofluorescence intensities in AAV9-injected mouse hippocampus, cerebral cortex, and cerebellum ($n = 6$). Relative GFP and relative FLAG immunofluorescence are expressed as signal intensities per unit area quantified by NIH ImageJ software. (**E**) Relative $\alpha 1$ ACT mRNA expression in the cerebellum of AAV9-injected mice at 4 weeks of age ($n = 4$). (**F**) Western blot and densitometric analyses showing $\alpha 1$ ACT-Q33-FLAG expression in the cerebellum of AAV9-injected mice at 4 weeks of age ($n = 4$). (**G** and **H**) Amount of miR-3191-5p (**G**) and $\alpha 1$ ACT-Q33 (**H**) mRNA binding to Ago4 that was immunoprecipitated using an antibody against Ago4 or a control IgG in the cerebellum of AAV9-Q33-miR-3191-5p mice at 4 weeks of age ($n = 6$). GFP, AAV9-GFP mice; mock, AAV9-Q33-miR-mock mice; 3191, AAV9-Q33-miR-3191-5p

mice. All data represent means $\pm$ SEM. ** $P < 0.01$. One-way ANOVA in (C). Student's t test in (D) to (H).

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Fig. 7. miR-3191-5p prevents Purkinje cell degeneration caused by *CACNA1A* IRES-driven $\alpha 1\text{ACT}_{\text{SCA6}}$ in mice

(A) Representative immunofluorescence images of AAV9-injected mouse cerebellum from 4-week-old mice stained with calbindin-specific antibodies and fluorescent secondary antibody. Scale bar, 100 μm . (B to D) Molecular layer thickness (B), density of dendritic tree (C), and Purkinje cell counts (D) in AAV9-injected mouse cerebellum from 4-week-old mice ($n = 6$). GFP, AAV9-GFP mice; mock, AAV9-Q33-miR-mock mice; 3191, AAV9-Q33-miR-3191-5p mice. All data represent means $\pm$ SEM. ** $P < 0.01$. Student's t test in (B) to (D).

Fig. 8. AAV9-miR-3191-5p prevents the ataxia and motor deficits caused by *CACNA1A* IRES-driven $\alpha 1ACT_{SCA6}$ in mice

(A to E) Clinical features of AAV9-injected mice. Body weight (A), rotarod test (B), and open-field assay (C) at 4 weeks of age. Stride length (D) and stride frequencies (E) assessed by DigiGait analysis at 4 weeks of age ($n = 12$, 6 male and 6 female mice per group). GFP, AAV9-GFP mice; mock, AAV9-Q33-miR-mock mice; 3191, AAV9-Q33-miR-3191-5p mice. All data represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. Two-way ANOVA in (A) and (B). Student's t test in (C) to (E).