Early death of ALS-linked CHCHD10-R15L transgenic mice with central nervous system, skeletal muscle, and cardiac pathology

HIGHLIGHTS

Transgenic mice expressing wild-type or ALS-linked CHCHD10 p.R15L developed CHCHD10-R15L mice display widespread axonal swellings in the CNS Mice perform well in motor tests despite CNS, skeletal muscle, and cardiac pathology Early death of CHCHD10-R15L mice likely due to cardiac failure
Early death of ALS-linked CHCHD10-R15L transgenic mice with central nervous system, skeletal muscle, and cardiac pathology

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SUMMARY
Mutations in coiled-coil-helix-coiled-coil-helix domain containing 10 (CHCHD10) have been identified in patients suffering from various degenerative diseases including mitochondrial myopathy, spinal muscular atrophy Jokela type, frontotemporal dementia, and/or amyotrophic lateral sclerosis (ALS). The pathogenic mechanism underlying CHCHD10-linked divergent disorders remains largely unknown. Here we show that transgenic mice overexpressing an ALS-linked CHCHD10 p.R15L mutation leads to an abbreviated lifespan compared with CHCHD10-WT transgenic mice. The occurrence and severity of the phenotype correlates to transgene copy number. Central nervous system (CNS), skeletal muscle, and cardiac pathology is apparent in CHCHD10-R15L transgenic mice. Despite the pathology, CHCHD10-R15L transgenic mice perform comparably to control mice in motor behavioral tasks until very close to death. Although paralysis is not observed, these models provide insight into the pleiotropic nature of CHCHD10 and suggest a contribution of CNS, skeletal muscle, and cardiac pathology to CHCHD10 p.R15L-ALS pathogenesis.

INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the degeneration of upper and lower motor neurons leading to paralysis, and ultimately death, usually within 3 to 5 years of diagnosis (Brown and Al-Chalabi, 2017). The causes of ALS are mostly unknown; however, approximately 10% of cases are recognized as familial. The causative genetic mutation has been identified in approximately 60% of these familial ALS (FALS) cases. The most common genes identified to be mutated in ALS patients include SOD1, C9orf72, and FUS (Rosen et al., 1993; Dejesus-Hernandez et al., 2011; Renton et al., 2011; Vance et al., 2009; Kwiatkowski et al., 2009), with over twenty more genes implicated to a lesser extent. One such gene is coiled-coil-helix-coiled-helix domain containing 10 (CHCHD10). Mutations have been reported in CHCHD10 in individuals affected with ALS as well as in other degenerative diseases including mitochondrial myopathy, other motor neuron phenotypes such as the Jokela type of spinal muscular atrophy, and frontotemporal dementia (FTD), among others (Bannwarth et al., 2014; Ajroud-Driss et al., 2015; Muller et al., 2014; Johnson et al., 2014; Jiao et al., 2016; Penttila et al., 2015; Kurzwelly et al., 2015; Project Mine ALS Sequencing Consortium, 2018; Auranen et al., 2015; Zhang et al., 2015; Lehmer et al., 2018). CHCHD10 is understood to encode for a mitochondrial protein. This could provide a direct etiological link between mitochondrial dysfunction and ALS pathogenesis.

CHCHD10 is so named due to the presence of two coiled coil helices, which each contain two cysteines separated by any nine amino acids (CX9C). The disulfide relay system facilitates the import of a CHCHD10 protein into the mitochondrial intermembrane space (Fischer and Riemer, 2013; Riemer et al., 2011; Herrmann and Riemer, 2012). The function(s) of CHCHD10 continues to be investigated. Proposals include, but are not limited to, a role for CHCHD10 in the mitochondrial contact site and cristae organizing system (MICOS) (Genin et al., 2016) as has been described for CHCHD3 and CHCHD6 (Darshi et al., 2012; Ott et al., 2015; An et al., 2012), although conflicting data questions this role (Huang et al., 2018; Burstein et al., 2018).

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CHCHD10 has also been proposed as a regulator of the efficiency of action of the electron transport chain (Burstein et al., 2018; Straub et al., 2018; Purandare et al., 2018) and as a biorganelar protein with a nuclear role in the regulation of gene transcription (Purandare et al., 2018). The latter function is similar to that proposed for CHCHD2 (Aras et al., 2015), mutations of which have been identified in familial Parkinson disease (PD) patients (Funayama et al., 2015). Expression of both CHCHD10 and CHCHD2 has been shown to be sensitive to oxygen tension (Purandare et al., 2018), and loss of one impacts the expression level of the other (Purandare et al., 2018; Huang et al., 2018). Protein-protein interactions and functional redundancy have been observed between CHCHD10 and CHCHD2 (Huang et al., 2018; Burstein et al., 2018; Straub et al., 2018).

Investigations examining the impact of knockdown (KD) or knockout (KO) of CHCHD10/CHCHD2 orthologs in C. elegans, zebrafish, and drosophila have been performed, and a variety of phenotypes were observed (Woo et al., 2017; Brockmann et al., 2018; Meng et al., 2017). Often, rescue was accomplished with introduction of the human wild-type CHCHD10 or CHCHD2 but not disease-linked mutations leading the authors to propose loss-of-function mechanisms of disease. It must be noted that a phenotype arising due to KD or KO of these orthologs cannot be attributed to loss of either CHCHD10 or CHCHD2 specifically, due to their homology. The C. elegans ortholog shares ~41% amino acid identity with CHCHD10 and CHCHD2. The zebrafish ortholog shares ~65% and ~57% amino acid identity with CHCHD10 and CHCHD2, respectively. The drosophila ortholog shares ~37% and ~48% amino acid identity with CHCHD10 and CHCHD2, respectively. The discordance in protein homology is amplified in the N-terminus, making it difficult to assess the impact of introduction of the human CHCHD10 p.R15L mutant, in particular, into these model systems.

Murine Chchd10 shares ~85% amino acid identity with human CHCHD10 and ~51% amino acid identity with human CHCHD2. Short-term studies of Chchd10-KO mice do not reveal a striking phenotype (Burstein et al., 2018; Liu et al., 2020b; Anderson et al., 2019). However, Chchd10/Chchd2 double KO mice display a variety of phenotypes including vacuolar pathology and abnormal mitochondrial cristae structure in the heart along with echocardiographic deficits (Liu et al., 2020b). Furthermore, the double KO mice display increased OMA1 cleavage of OPA1 and upregulation of the mitochondrial integrated stress response. The same study reported similar deficits in Chchd10 p.S55L knock-in (KI) mice, which model the human CHCHD10 p.S55L mutation that was identified in patients with complex and varying phenotypes including myopathy, ataxia, cognitive impairment, motor neuron disease and deafness, as well as a singleton ALS-FTD case (Bannwarth et al., 2014). Combined, these data lead the authors to propose that aggregation prone CHCHD10 p.S55L may act through a dominant negative mechanism by pulling soluble CHCHD10 and CHCHD2 into an insoluble fraction, thereby mimicking the double KO mice. Alternatively, or additionally, the insoluble fraction may act through a toxic gain-of-function mechanism. Further investigation is required to distinguish between the two mechanisms.

Chchd10 p.S55L KI mice were previously developed by two other independent groups (Anderson et al., 2019; Genin et al., 2019). Both groups report a survival of ~330 days. One group reports progressive motor deficits, myopathy, cardiomyopathy, Chchd10 aggregation, and upregulation of the mitochondrial integrated stress response (Anderson et al., 2019). None of these phenotypes were observed in Chchd10-KO mice, leading the authors to propose a toxic gain-of-function mechanism of action of Chchd10 p.S55L but cautioned that loss-of-function or dominant negative mechanisms may still play a role in human disease manifestation. The second group report a variety of mitochondrial ultrastructure, mitochondrial DNA, and respiratory chain deficiencies in muscle, heart, brain, and spinal cord (Genin et al., 2019). The authors propose that early myopathic changes precede central nervous system involvement. A conditional skeletal muscle knockout mouse model has also been developed and displays some mild motor deficits, along with neuromuscular junction structural and electrophysiological deficits (Xiao et al., 2019).

Combined, these data from in vivo models highlight the importance of functional redundancy between CHCHD10 and CHCHD2, the tissue-specific impact of mutations, and that the underlying mechanism of disease for individual mutations remains to be fully elucidated.

As part of efforts to identify genetic causes of ALS, we identified a CHCHD10 p.R15L mutation in five FALS pedigrees (Manuscript in preparation). This mutation has also been identified by five other independent research groups and is likely responsible for <1% of FALS cases (Muller et al., 2014; Kurzwelly et al.,
Figure 1. Development and survival analysis of CHCHD10 transgenic mice

(A) 6.4kb human CHCHD10 transgene design indicating restriction enzyme cutting sites and exons 1–4. Gray boxes represent untranslated regions. Black boxes represent translated regions.

(B) Electropherograms displaying the nucleotide sequences of CHCHD10-WT and CHCHD10-R15L transgenes identified in mouse tail DNA. The pathogenic G > T change leading to the R15L mutation is indicated in pink. A C > A change in the CHCHD10-R15L lines, indicated by the overlaying asterisk, is a reported synonymous SNP in humans (rs179468) that was also introduced during site-directed mutagenesis.

(C) Agarose gel displaying PCR amplification products of indicated mouse tail DNA using primers directed against human CHCHD10 and murine Actb. A non-transgenic mouse tail DNA sample and H2O sample are used as negative controls, demonstrating the specificity of the primers used. The relative transgene copy number is demonstrated qualitatively.

(D) Western blot analysis of transgene expression at the protein level. Mouse forebrain lysate was prepared using RIPA buffer, and immunoblotting was carried out using the indicated antibodies.

(E) Quantitation of transgenic human CHCHD10 protein expression in indicated mouse lines from n = 3 independent experiments. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, *p < 0.05, **p < 0.001.

(F) Kaplan-Meier survival analysis of the indicated CHCHD10 founder lines. Death of a mouse for unknown reasons is considered a death event, as indicated by a drop in the line. Mice still living or death of a mouse due to intervention by the investigators are considered censored events, as indicated by tick marks along a line. Median survival for CHCHD10-WT (L) is 921 days, n = 12 death events and n = 76 censored events. Median survival for CHCHD10-WT (H) is 1,003 days, n = 7 death events and 51 censored events. Gehan-Breslow-Wilcoxon test, p = 0.1847 in comparison to CHCHD10-WT (L) line. Median survival for CHCHD10-R15L (L) is 888 days, n = 11 death events and 83 censored events. Gehan-Breslow-Wilcoxon test, p = 0.5900 in comparison to CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, p = 0.5879 in comparison to CHCHD10-WT (H) line. Median survival for CHCHD10-R15L (M) is 691 days, n = 24 death events and n = 107 censored
In the current study, we sought to investigate ALS pathogenesis caused by the CHCHD10 p.R15L mutation. We undertook the approach of engineering transgenic mouse models expressing the human CHCHD10 gene with, and without, the p.R15L mutation under the control of the endogenous CHCHD10 gene promoter. We have observed a range of striking pathologies of the CNS, skeletal muscle, and heart in CHCHD10-R15L transgenic mice that result in an abbreviated lifespan in a copy-number-correlated fashion.

RESULTS
Development of CHCHD10 transgenic mouse lines
We constructed a 6.4kb KpnI/BamHI human CHCHD10 genomic DNA transgene using a human genomic DNA BAC clone (RP11-124F9, 180.7kb) as a template. The transgene was assembled by ligation of three fragments (Figure 1A). The transgene includes the promoter region of 3.4kb upstream of the CHCHD10 transcription starting site, all four exons and three introns of CHCHD10, and 0.7kb fragment downstream of the CHCHD10 poly(A) signal (Figure 1A). The ALS-linked CHCHD10 p.R15L mutation was introduced by site-directed mutagenesis (Figure 1B). A reported synonymous SNP in humans (rs179468) was also introduced to the CHCHD10-R15L construct during site-directed mutagenesis. Transgenic mouse lines were established by microinjection of the transgene into fertilized eggs. Analysis of the transgene copy number and transgenic protein expression levels indicated that the CHCHD10-WT (L) line had significantly lower transgene copy number and expression than that of the CHCHD10-R15L lines (Figures 1C–1E). We subsequently performed another round of transgene microinjection and identified a mouse line, CHCHD10-WT (H), with a transgene copy number and transgenic protein expression level higher than that of the low-expressing CHCHD10-R15L (L) line.

The transgenic mouse lines examined in this study include a low-expressing CHCHD10-WT (L) line, a high-expressing CHCHD10-WT (H) line, a low-expressing CHCHD10-R15L (L) line, a medium-expressing CHCHD10-R15L (M) line, and a high-expressing CHCHD10-R15L (H) line (Figures 1C–1E). The CHCHD10-R15L (H) line is particularly difficult to breed, as demonstrated by a reduced litter size at P21 compared with other genotypes, and only 22% of mice weaned at P21 being transgenic, compared with the expected 50% (Figure S1). These data do not account for death of mice or cannibalism by a parent before P21. Strikingly, CHCHD10-R15L (M) and CHCHD10-R15L (H) transgenic mice die unexpectedly before the CHCHD10-WT (L) and CHCHD10-WT (H) mice in a copy-number-correlated fashion (Figures 1F, S2A, and S2B).

Behavioral and phenotypic testing reveal differences between CHCHD10-WT and CHCHD10-R15L lines
A cohort of mice from the CHCHD10-WT (L), CHCHD10-WT (H), and CHCHD10-R15L (M) lines was monitored for body weight and rotarod performance in a longitudinal study in order to examine the general health and motor ability of the transgenic mouse lines. Mice were tested every 60 days, beginning at 60 days of age. Rotarod performance is comparable between the lines available up to the 600-day time point (Figures 2A, S2C, and S2D, Tables S1–S18). Following on from this, there is a combination of early death of CHCHD10-R15L (M) mice and a precipitous decline in the rotarod performance of those surviving. These phenomena are preceded by a notable loss of body weight compared with the CHCHD10-WT (L) mice, although body weight is similar between CHCHD10-WT (H) and CHCHD10-R15L (M) mice (Figures 2B and 2C, Tables S19–S30). The motor behavior of separate cohorts of 4-month-old and 14- to 20-month-old mice was monitored in an open field test. All genotypes traveled similar distances at similar velocities over the course of the experiment (Figures 2D–2G). As an approximate measure of anxiety-like behavior, all genotypes of mice spent an equivalent length of time in the periphery and center of the open field at both ages (Figures 2H–2K) (Seibenhener and Wooten, 2015; Simon et al., 1994). DigiGait analysis reveals few statistically significant gait disturbances between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic mice (Tables S31 and S32), with the vast majority of parameters tested equivalent between the two lines at both ages. We noted that a greater number of 20-month-old CHCHD10-R15L (M) mice were...
Figure 2. Behavioral testing of CHCHD10 transgenic mouse lines

(A) Mouse rotarod performance. Mice were tested on an accelerating rotarod task every 60 days beginning at 60 days of age until all CHCHD10-R15L (M) mice in the cohort died. The overlying numbers indicate the number of mice from each line used to calculate mean latency to fall at each time point. Data are represented as mean ± SEM. Two-way ANOVA with Holm–Šidák post hoc statistical analysis summarized in Tables S1–S6.

(B) Male mouse body weight measurements. Mouse body weight was measured every 60 days beginning at 60 days of age until all male CHCHD10-R15L (M) mice in the cohort died. The overlying numbers indicate the number of mice from each line used to calculate mean body weights at each time point. Data are represented as mean ± SEM. Two-way ANOVA with Holm–Šidák post hoc statistical analysis summarized in Tables S25–S30.

(C) Female mouse body weight measurements. Mouse body weight was measured every 60 days beginning at 60 days of age until all female CHCHD10-R15L (M) mice in the cohort died. The overlying numbers indicate the number of mice from each line used to calculate mean body weights at each time point. Data are represented as mean ± SEM. Two-way ANOVA with Holm–Šidák post hoc statistical analysis summarized in Tables S19–S24.

(D) The mean distance traveled by 4-month-old mice of the indicated lines in an open field test over five minutes. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

(E) The mean distance traveled by 14- to 20-month-old mice of the indicated lines in an open field test over 5 minutes. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

(F) The mean velocity of 4-month-old mice of the indicated lines in an open field test over 5 minutes. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

(G) The mean velocity of 14- to 20-month-old mice of the indicated lines in an open field test over 5 minutes. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

(H) The mean percentage time spent in the center of the open field by 4-month-old mice of the indicated lines. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.
Figure 2. Continued

(I) The mean percentage time spent in the periphery of the open field by 4-month-old mice of the indicated lines. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

(J) The mean percentage time spent in the center of the open field by 14- to 20-month-old mice of the indicated lines. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

(K) The mean percentage time spent in the periphery of the open field by 14- to 20-month-old mice of the indicated lines. Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05.

Unable to complete the task at higher belt speeds compared with CHCHD10-WT (L) mice (Table S33). However, those mice unable to perform at higher belt speeds performed, comparably to CHCHD10-WT (L) mice, at slower belt speeds, demonstrating their ability to execute the movement but their sensitivity to the intensity of the task. A loss of motivation to perform the task at higher belt speeds could also arise due to cognitive impairment, rather than the development of motor deficits. These behavioral tests of motor function demonstrate that CHCHD10-R15L (M) mice perform, comparably to controls, until close to the point of death. Paralysis was never observed to occur before death.

Profound central nervous system pathology of CHCHD10-R15L transgenic mice

Given the progressive behavioral deficits apparent in the CHCHD10-R15L (M) transgenic mice, we analyzed the pathology of the central nervous system at various timepoints. When using either commercial or in-house designed antibodies directed against CHCHD10-specific epitopes, we observe a remarkable and widespread pathology in CHCHD10-R15L transgenic mice characterized by abnormal swellings along neuritic processes (Figures 3A–3J, S3, and S4). These swellings often contain a strongly immunoreactive component, localized to a focal point on the periphery of the swelling. The CNS pathology is present in all three lines, including the lowest expressing line, although the pathology is more florid in the higher expressers (Figure 3K). This pathology first becomes apparent at 60 days of age in CHCHD10-R15L (M) mice (Figure S6). The pathology appears to arise due to the CHCHD10-R15L mutation, rather than an artifact of transgene overexpression as it is absent from both CHCHD10-WT (L) and CHCHD10-WT (H) transgenic mouse lines and is present in all CHCHD10-R15L lines, regardless of copy number. Given the reported mitochondrial and nuclear localization of CHCHD10, this immunohistochemical profile is surprising, as it does not reflect a typical mitochondrial or nuclear staining profile.

In order to determine the nature of the neuritic processes harboring these abnormal swellings, we analyzed the colocalization of either dendritic or axonal markers with CHCHD10. This revealed an overlap between CHCHD10 and neurofilament markers (SMI 32, SMI 310R, and NF-68) but not Map2-positive dendrites (Figures 4A–4F, and S4A–S4F). Confirmation of the neuronal, and specifically axonal, location of these swellings was provided by ultrastructural analysis with transmission electron microscopy (Figures 4G, 4H, and S6A–S6F). This revealed myelinated axons harboring membrane-bound swellings with an amorphous matrix. The membrane lining is predominantly a single membrane with focal accumulations of membranous whorls protruding into the swelling visible in most cases. These are likely to be the strongly immunoreactive components observed by immunohistochemistry.

Given the known mitochondrial location of CHCHD10, we examined whether mitochondrial proteins contribute to any component of the axonal swellings. Firstly, the unusual staining pattern of long stretches of neuritic processes observed using various CHCHD10 antibodies was specific to those antibodies and not observed when using other antibodies directed against mitochondrial proteins. Interestingly, although the vast majority of the membranes of the swellings did not co-localize with other mitochondrial markers, the strongly immunoreactive CHCHD10-positive focal components often co-localized with mitochondrial markers. This co-localization was most prominent for inner mitochondrial membrane proteins, ATP synthase α subunit, and COX IV subunit Vb (Figures S4G–S4L).

By using the endogenous CHCHD10 gene promoter to drive expression of the transgene, we did not restrict expression to any particular known cell type. Indeed, the pathological swellings observed are present in axons of a variety of neurons throughout the CNS. To describe the CHCHD10-R15L (M) line in detail, the swellings are apparent in cholinergic motor neurons in the spinal cord anterior horn (Figures S5 and S7). Prominent swellings are also apparent in the anterior commissure of the spinal cord. They can be observed in all levels and laminae of the spinal cord gray matter, with a greater abundance in the anterior gray matter. Swellings are numerous and apparent in the brainstem of all CHCHD10-R15L transgenic mouse lines and to varying extents in other brain regions (Figure S8). We examined whether pathology extended to the
Figure 3. CNS pathology of CHCHD10-R15L transgenic mice

(A) A 20-month-old male CHCHD10-WT (L) anterior horn from one side of a transverse spinal cord section stained using an antibody targeting CHCHD10. Region within black box outline is magnified in (B). Scale bar: 100 μm.

(B) Higher magnification of region indicated in (A). Scale bar: 25 μm.
Figure 3. Continued

(C) A 35-month-old male CHCHD10-WT (H) anterior horn from one side of a transverse spinal cord section stained using an antibody targeting CHCHD10. Region within black box outline is magnified in (D). Scale bar: 100 μm.

(D) Higher magnification of region indicated in (C). Scale bar: 25 μm.

(E) A 10-month-old male CHCHD10-R15L (L) anterior horn from one side of a transverse spinal cord section stained using an antibody targeting CHCHD10. Region within black box outline is magnified in (F). Scale bar: 100 μm.

(F) Higher magnification of region indicated in (E) demonstrates the presence of neuritic processes with swellings along the length of the neuritic process. Example of a swelling is indicated by a black arrow. Scale bar, 25 μm.

(G) A 13-month-old male CHCHD10-R15L (M) anterior horn from one side of a transverse spinal cord section stained using an antibody targeting CHCHD10. Region within black box outline is magnified in (H). Scale bar, 100 μm.

(H) Higher magnification of region indicated in (G) demonstrates the presence many neuritic processes with swellings that appear either in isolation or in sequence along the length of the neuritic process. Examples of swellings are indicated by black arrows. Scale bar, 25 μm.

(I) A 14-month-old female CHCHD10-R15L (H) anterior horn from one side of a transverse spinal cord section stained using an antibody targeting CHCHD10. Region within black box outline is magnified in (J). Scale bar: 100 μm.

(J) Higher magnification of region indicated in (I) demonstrates the presence many neuritic processes with swellings along the length of the neuritic process. Examples of swellings are indicated by black arrows. Scale bar, 25 μm.

(K) Quantitation of the number of swellings in the right and left lumbar spinal cord anterior horn gray matter per section of 8- to 14-month-old mice (n = 3, m = 3/genotype). Data are represented as mean ± SEM. One-way ANOVA with Tukey’s multiple comparison test, ns p > 0.05, *p < 0.05, ***p < 0.001.

Peripheral nervous system by analyzing the numbers and caliber distribution of femoral nerve motor branch axons. No difference in axon number or axon caliber distribution was apparent between the transgenic lines examined (Figures S9A–S9D, Table S34). These data indicate that the CNS pathology does not result in a loss of motor neurons that give rise to peripheral motor axons.

Given the mitochondrial location of CHCHD10 and the CNS axonal pathology observed, we analyzed the movement of mitochondria in the axons of primary neurons derived from E12.5 embryonic spinal cords. This assay revealed a reduction in both the anterograde and the retrograde movement of mitochondria of CHCHD10-R15L (M) primary neurons compared with non-transgenic primary neurons derived from littermates (Figure S9E). Net movement of CHCHD10-R15L (M) mitochondria is in the retrograde direction, and this can be accounted for by a reduction in the percent of time mitochondria were observed moving anterogradely (Figure S9F). There was no difference in the mitochondrial density or basal oxygen consumption rate of these neurons, but there was a slight increase in the length of CHCHD10-R15L (M) mitochondria compared with non-transgenic mitochondria (Figures S9G–S9I). Taken together, these data suggest deficits in mitochondrial trafficking and dynamics of CHCHD10-R15L (M) mouse spinal cord neurons with the caveat that these deficits may arise due to transgene over-expression and/or the mutation.

Cytoplasmic p62-positive skein-like inclusions in motor neurons are a pathological hallmark of ALS. Loss of nuclear TDP-43 and cytoplasmic TDP-43 inclusions are a common pathological feature in ALS cases, with notable exceptions including, but not limited to, SOD1-ALS (Mackenzie et al., 2007). Although we do not observe cytoplasmic p62-positive skein-like inclusions in motor neurons, abundant punctate p62-positive immunoreactivity is apparent in the spinal cord gray matter of CHCHD10-R15L (M) and CHCHD10-R15L (H) mice (Figure S10). The identity of the cells and cellular compartments harboring these p62-positive puncta remains to be determined. Immunohistochemical staining of spinal cord using an antibody targeting TDP-43 appears typical with prominent nuclear immunoreactivity (Figure S10).

Skeletal and cardiac muscle pathology of CHCHD10-R15L transgenic mice

The relatively long life-span and unexpected death of CHCHD10-R15L transgenic mice over a wide time frame suggests cardiopulmonary failure as the immediate cause of death. Given the severe CNS pathology that is apparent at least from 60 days of age in CHCHD10-R15L (M) mice in axons of a wide array of neurons of diverse neuronal systems, the length of the lifespan, although abbreviated, is quite surprising. We observed, however, that the respiratory rate of the CHCHD10-R15L (M) mice appeared to be increased in the weeks before death. This led us to examine the muscular pathology of the diaphragm, which revealed some striking features (Figures S11D–S11F). There is an obvious increase of connective tissue and cellularity between myofibers. We expanded this analysis to include the quadriceps and gastrocnemius of other mouse lines and observe similar myopathic changes (Figures 5A–5F, and S11A–S11C). Many abnormal myofiber profiles are apparent by hematoxylin and eosin (H&E) staining such as short diameter fibers, cytoplasmic hematoxylin invasion with focal eosinophilic cores, and central nuclei. Upon immunohistochemical
analysis, we again observed an unusual staining profile when using antibodies targeting CHCHD10. Staining was absent from some myofibers. Other myofibers harbored CHCHD10-positive accumulations some of which, but not all, were p62-positive (Figures 6G–6N). Electron microscopy reveals large fibrous interruptions of unknown composition to the myofibrillary network (Figures S11G–S11J). Despite the severe pathology, high-resolution respirometry analysis of soleus or tibialis anterior muscles does not reveal any difference in the oxygen consumption of 7- to 8-month-old mice (Figures 6O and 6P), indicating that mitochondrial oxidative phosphorylation capacity is not compromised, at least at this relatively early stage in CHCHD10-R15L (M) transgenic mice skeletal muscle.

We carried out an echocardiography study of younger and older cohorts of mice to determine whether any cardiac impairment might contribute to the early death of CHCHD10-R15L transgenic mice. No statistically significant difference was observed in any parameter tested, including, but not limited to, cardiac output,
ejection fraction, fractional shortening, left ventricular mass, and stroke volume in the younger cohort (Figures 6A–6F, 6J, and 6K). Although limited in number, we carried out an echocardiography study of an older cohort of available mice (Figures 6G–6I, 6L, and 6M). Fourteen- to sixteen-month-old CHCHD10-R15L (H) transgenic mice (Figures 6G–6I, 6L, and 6M) were examined to determine if there were any age-related changes in cardiac function.

**Figure 5. Widespread myopathic abnormalities in CHCHD10-R15L transgenic mice**

(A–F) Hematoxylin and eosin staining of quadriceps of a 22-month-old female non-transgenic mouse (A), a 5-month-old male CHCHD10-WT (L) mouse (B), a 14-month-old female CHCHD10-WT (H) mouse (C), a 23-month-old female CHCHD10-R15L (L) mouse (D), a 15-month-old female CHCHD10-R15L (M) mouse (E), and a 12-month-old male CHCHD10-R15L (H) mouse (F). Abundant myopathic features are apparent in the CHCHD10-R15L transgenic mice including central nuclei, hematoxylin invasion, eosinophilic cores, and increased intermyofibrillary cellularity. Milder myopathic changes are apparent in CHCHD10-WT (H) skeletal muscle with occasional central nuclei. Scale bars, 50 μm.

(G–N) Immunofluorescence of 14-month-old female CHCHD10-WT (H) and 15-month-old female CHCHD10-R15L (M) transgenic mouse quadriceps using antibodies targeting CHCHD10 and p62. Transgenic CHCHD10 protein expression is somewhat mosaic in appearance and often shares immunoreactivity with p62-positive inclusions. Scale bars, 25 μm. (J, K) Muscle respirometry of permeabilized fibers from tibialis anterior (O) or soleus (P) from 7- to 8-month-old male mice (n = 3/group) demonstrating the oxygen consumption of the muscle fibers under sequential substrate, uncoupler, and inhibitor application conditions, as indicated. Data are represented as mean ± SEM. Unpaired t test, ns p > 0.05.

Figure 5. Widespread myopathic abnormalities in CHCHD10-R15L transgenic mice.
| Short Axis B-Mode | Diastole | Systole | Short Axis M-Mode |
|------------------|---------|---------|------------------|
| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
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**Figure 6.** Echocardiography demonstrates transgene copy number and age-related decline in cardiac function

(A–C) Representative short axis echocardiography views from a 6-month-old male CHCHD10-WT (L) transgenic mouse.

(D–F) Representative short axis echocardiography views from a 7-month-old male CHCHD10-R15L (M) transgenic mouse.

(G–I) Representative short axis echocardiography views from a 16-month-old male CHCHD10-R15L (H) transgenic mouse.

(J) Echocardiography calculations derived from short axis M-Mode measurements of younger female mice. n = 3 6- to 7-month-old CHCHD10-WT (L) mice; n = 3 7- to 9-month-old CHCHD10-R15L (M) mice. Data are represented as mean ± SEM. Unpaired t test, ns p > 0.05.

(K) Echocardiography calculations derived from short axis M-Mode measurements of younger male mice. n = 3 6- to 7-month-old CHCHD10-WT (L) mice; n = 3 6- to 7-month-old CHCHD10-R15L (M) mice. Data are represented as mean ± SEM. Unpaired t test, ns p > 0.05.

(L) Echocardiography calculations derived from short axis M-Mode measurements of older female mice. n = 3 18-month-old CHCHD10-WT (L) mice; n = 3 18-month-old CHCHD10-R15L (M) mice; n = 1 15-month-old CHCHD10-R15L (H) mouse. Data are represented as mean ± SEM. Unpaired t test, ns p > 0.05.
mice displayed a statistically significant increased left ventricular mass compared with CHCHD10-WT (H) mice. Included in this study was a 16-month-old male CHCHD10-R15L (H) transgenic mouse that displayed an increased respiratory rate around the time of testing. This mouse had a reduced ejection fraction (22%) and fractional shortening (10%), severe ventricular hypokinesis (Figures 6G–6I), as well as an increased left ventricular mass (240mg) indicating a cardiomyopathy and cardiac failure. The mouse died 10 days later around the median age of death for this line. The onset of cardiac failure appears to be sudden as four other 14- to 16-month-old mice of the same gender and genotype did not display functional deficits as severe as the 16-month-old mouse. Furthermore, heart weight:body weight ratios were comparable across all lines, indicating that cardiac hypertrophy is not a factor at the ages measured (Figure 6N).

We also examined the cardiac pathology of the transgenic mouse lines. All CHCHD10-R15L transgenic lines display degenerating cardiomyocytes with CHCHD10-positive, p62-positive, and ubiquitin-positive accumulations (Figures 7A–7U). This pathology appears to be correlated with age and transgene copy number, similar to the previously described pathologies. A similar mild pathology is apparent in the CHCHD10-WT (H) mice. Unlike the CNS pathology, the possibility remains that transgenic protein overexpression is a causative factor giving rise to this pathology rather than the mutation, because it is apparent in the CHCHD10-WT (H) mice to a limited extent. The current data do not facilitate resolving these possibilities; nevertheless, the pathology and echocardiography data suggest that CHCHD10-R15L mice die suddenly from cardiac failure due to the degeneration of cardiomyocytes.

Chchd10 knockout mice

In order to address whether the pathology and deficits observed in CHCHD10-R15L transgenic mice occur as a result of a toxic gain- or loss-of-function, we analyzed mice lacking murine Chchd10 (Figures S12A–S12C). A cohort of Chchd10 homozygous knockout (KO) and control C57BL/6J mice was monitored for survival, body weight, and rotorod performance in a longitudinal study (Figure 8). Little difference was observed between the lines at any age tested. Furthermore, the axonal swelling pathology and cardiac pathology observed in the CHCHD10-R15L transgenic lines are absent from the Chchd10-KO mice (Figures S12D–S12G). A recent report describing Chchd10/Chchd2 double KO mice describes vacuolar pathology and abnormal mitochondrial cristae structure in the heart along with echocardiographic deficits resulting in early death of the mice (Liu et al., 2020b). The discordance between the single and double KO mice suggests Chchd2 compensates for the lack of Chchd10 in the single KO mice. The cardiac phenotype displays some similarity with the CHCHD10 transgenic mice; however, no CNS pathology was reported in the double KO mice. Although mutant CHCHD10 might act through a toxic gain-of-function mechanism in the heart, these data also raise the possibility that mutant CHCHD10 might act through a combination of loss of function of mutant CHCHD10 and a dominant negative mechanism of action on wild-type CHCHD10 and CHCHD2 to prevent compensation, resulting in a cardiac phenotype. CHCHD10 p.R15L appears to act through a toxic gain-of-function mechanism to give rise to the CNS pathology apparent in CHCHD10-R15L transgenic mice, because it is absent from CHCHD10-WT transgenic mice and Chchd10-KO mice and not reported in Chchd10/Chchd2 double KO mice.

In summary, CHCHD10-R15L transgenic mice display age- and copy-number-correlated pathologies of the CNS, skeletal muscle, and heart. These pathologies precede premature death and provide an insight into the mechanisms of disease at play in CHCHD10 p.R15L-ALS.

DISCUSSION

We report here the development of transgenic mouse models that provide useful tools to study the pathogenesis of CHCHD10 p.R15L-ALS. Mutations in CHCHD10 have been identified in individuals suffering from distinct diseases of the nervous and muscular systems. This highlights the pleiotropic nature of CHCHD10 in that it can impact upon distinct phenotypes. These transgenic mouse models support the
hypothesis that CHCHD10 is a pleiotropic gene and suggest that a combination of tissue-specific pathologies may act in concert to cause disease in CHCHD10 p.R15L-ALS.

One must be cautious in interpreting data from transgenic mouse models due to confounding factors related to overexpression, position effects, and variable expression levels across models. In addition to these confounding factors, animal models may have limitations due to species differences and their short lifespan, which is particularly relevant to late-onset neurodegenerative disease. We took the approach of developing these models because transgenic overexpression of mutant genes that cause late-onset disorders is typically employed to exaggerate the toxicity of the mutant protein and thus compensate for the short lifespan of the model. The shared pathological phenotypes across multiple independent lines suggests position effects are not a major concern. We are mindful of the impact and potential confounding of transgene overexpression. It is for this reason that we established and characterized five independent transgenic mouse lines, as described. We screened 66 potential CHCHD10-WT founder mice generated over two rounds of microinjections in order to obtain a line with comparable levels of expression to the CHCHD10-R15L lines that have a range of transgene expression levels. Given the limitations of the technology in that we could not control levels of transgene expression with exquisite precision, we proceeded with the best available lines. A recent report describes the development of a transgenic mouse model expressing FLAG-tagged CHCHD10 under the control of the CNS-enriched Prp promoter (Liu et al., 2020a). FLAG-tagged CHCHD10-R15L transgenic mice display one-half the transgene expression level of the FLAG-tagged CHCHD10-WT control mice, again highlighting the inherent difficulties of obtaining transgenic mice with comparable transgene expression levels. The authors describe a disruption of the interaction between CHCHD10, Opa1, and mitofilin, a component of the MICOS system, in the mutant mice. No behavioral, pathological, or survival data are described, making it difficult to compare the models.

The FLAG-tagged CHCHD10 transgenic mouse model contrasts with the transgenic mouse models we describe in that we did not restrict expression to a particular cell type or tissue. The transgene design, incorporating the entire human CHCHD10 gene and endogenous upstream promoter, has several favorable features. Use of the endogenous promoter allows for analysis of the impact of expression on the organism as a whole, without restriction to a specific cell type or tissue, thus providing a means of discerning the pleiotropic properties of CHCHD10. Furthermore, the availability of the human gene sequence in a mouse model provides a means to perform preclinical studies targeting the human sequence in vivo, an approach undertaken leading to ongoing investigations related to SOD1-ALS therapeutic development (McCampbell et al., 2018; Borel et al., 2016).

ALS is a genetically, clinically, and pathologically complex disease. With the discovery of mutations in genes such as SOSTM1, C9orf72, VCP, HNRNPA1, and HNRNPA2/B1 in ALS patients (Fecto et al., 2011; Dejesus-Hernandez et al., 2011; Renton et al., 2011; Johnson et al., 2010; Kim et al., 2013), it has come to be recognized that ALS can fall within a spectrum of clinical presentations. These genes can also be mutated in patients suffering from the individual diseases or various combinations of frontotemporal dementia, myopathy, and/or Paget disease of bone (Dejesus-Hernandez et al., 2011; Renton et al., 2011; Kim et al., 2013; Watts et al., 2004). A spectrum of ALS pathologies has also become apparent with the identification of causative gene mutations. For example, SOD1-FALS cases are not typically observed to harbor FUS, TDP-43, or OPTN-positive protein inclusions, as is the case for many sporadic ALS and...
Figure 8. Chchd10-KO mouse behavioral testing

(A) Male mouse body weight measurements. Mouse body weight was measured every 60 days beginning at 60 days of age. The overlying numbers indicate the number of mice from each line used to calculate mean body weights at each time point. Data are represented as mean ± SEM. Two-way ANOVA with Holm–Šidák post hoc statistical analysis summarized in Tables S35–S36.

(B) Female mouse body weight measurements. Mouse body weight was measured every 60 days beginning at 60 days of age. The overlying numbers indicate the number of mice from each line used to calculate mean body weights at each time point. Data are represented as mean ± SEM. Two-way ANOVA with Holm–Šidák post hoc statistical analysis summarized in Tables S37–S38.

(C) Kaplan-Meier survival analysis of Chchd10-KO mice compared with C57BL/6J mice. Death of a mouse for unknown reasons is considered a death event, as indicated by a drop in the line. Mice still living or death of a mouse due to intervention by the investigators are considered censored events, as indicated by tick marks along a line. Median survival for Chchd10-KO mice is 851 days, n = 8 death events and n = 40 censored events. Median survival for C57BL/6J mice is undefined, n = 0 death events and n = 94 censored events. Gehan-Breslow-Wilcoxon test, p = 0.0107.

(D) Mouse rotarod performance. Mice were tested on an accelerating rotarod task every 60 days beginning at 60 days of age. The overlying numbers indicate the number of mice from each line used to calculate mean latency to fall at each time point. Data are represented as mean ± SEM. Two-way ANOVA with Holm–Šidák post hoc statistical analysis summarized in Tables S39–S40.
non-SOD1-FALS cases (Deng et al., 2010, 2011; Tan et al., 2007; Mackenzie et al., 2007). Despite the pathological diversity, these cases may be clinically indistinguishable. It is with this in mind that we observe an array of striking pathologies of the CNS, skeletal muscle, and heart in CHCHD10-R15L transgenic mice. Although these pathologies may not be recognized as a component of the typical pathological profile of ALS, they may be relevant to CHCHD10-ALS.

A recent description of the human pathology of CHCHD10 p.R15L-ALS describes anterior horn neuronal CHCHD10-positive, TDP-43-negative protein aggregates (Keith et al., 2020). Such aggregates are not apparent in the CHCHD10-R15L transgenic mice, but p62-positive, TDP-43-negative punctate staining is apparent in the spinal cord, indicating that the mice display some deficit in CNS protein degradation. Although axonal swelling, as observed in the CHCHD10-R15L transgenic mice, is not described in the human case, such pathology may not be readily observed due to species differences, limitations of detection, or prior loss of pathological axons.

By virtue of the presence of a coiled-coil-helix-coiled-coil-helix domain in CHCHD10, and the involvement of such a domain in the disulfide relay system to maintain proteins within the mitochondrial intermembrane space (Herrmann and Riemer, 2012; Modjtahedi et al., 2016), CHCHD10 is understood to be a mitochondrial protein. Work from a number of research groups have demonstrated CHCHD10 localization to mitochondria (Bannwarth et al., 2014; Ajroud-Driss et al., 2015; Huang et al., 2018; Burstein et al., 2018; Straub et al., 2018; Woo et al., 2017), as well as to the nucleus (Purandare et al., 2018). CHCHD10 is highly expressed in skeletal muscle and the heart, tissues known to have a relatively large mitochondrial population. Using the endogenous CHCHD10 promoter in our transgenic models, we did not restrict expression and subsequently detected severe pathologies of tissues where CHCHD10 is known to be abundant, i.e. skeletal muscle and heart, as well as in subpopulations of CNS neurons.

The CNS pathology is characterized by large axonal swellings that can occur in isolation or in sequence along an axon. The CNS pathology is apparent in all three CHCHD10-R15L transgenic lines, regardless of transgene copy number and never observed in either CHCHD10-WT transgenic line. This provides evidence that it arises due to the mutation and not as a result of transgene overexpression. A similar CNS pathology has been described in other transgenic mouse models related to ALS expressing both wild-type SOD1 and SOD1 p.G93A (Dal Canto and Gurney, 1997; Wong et al., 1995; Jaarsma et al., 2000, 2001; Tu et al., 1996). These models demonstrate mitochondrial vacuolation leading to swellings in axons of diverse CNS neurons akin to what we observe in CHCHD10-R15L transgenic mice. It is also noted that these swellings do not necessarily lead to neuronal death in SOD1-WT transgenic mice, as also appears to be the case in CHCHD10-R15L mice given that they survive many months, even years, after the first presentation of the pathological swellings. Given that neurons harboring swellings do not appear to degenerate, the possibility arises that such swellings might be protective against unrecognized pathogenic mechanisms or the life span of the mouse is not sufficiently long for the pathology to manifest as clinical disease.

The immunohistochemical profile visualized in the CNS when using antibodies targeting CHCHD10 is surprising in CHCHD10-R15L transgenic mice. Immunoreactivity is not obviously mitochondrial or nuclear but apparent in the pathological swellings and long stretches of axonal processes. The observation of this immunohistochemical profile with all independent antibodies used and lack of staining in Chchd10-KO mice indicates that this is an accurate representation of transgenic CHCHD10 protein localization. Focal components of the swellings that are strongly immunoreactive with CHCHD10 antibodies also display immunoreactivity with antibodies targeting mitochondrial proteins. This suggests that compromised
mitochondria contribute to the formation of these swellings. Indeed, profiles of swollen mitochondria with distended cristae, likely to be in an intermediate pathological stage leading toward this eventuality, are apparent (Figure S6F). Although pathological, these swellings do not appear to compromise cell viability, with indirect evidence provided by equivalent numbers and caliber distribution of femoral nerve motor branch axons. This highlights the resilience of neuronal systems to severe pathology, an underappreciated aspect of neuropathology. The mechanisms at play in driving the formation of the swellings remain to be fully determined. Plausible possibilities include deficits of mitochondrial dynamics, and our examination of mitochondrial transport in axons of primary neurons of CHCHD10-R15L (M) mice suggest mitochondrial trafficking abnormalities might contribute.

Given the severity of the CNS pathology including of motor system neurons, it is remarkable that CHCHD10-R15L transgenic mice perform, comparably to controls, in motor behavioral tests for the vast majority of life. Because the pathology is first apparent around 60 days of age in CHCHD10-R15L (M) mice, but they do not display any motor behavior deficit for approximately another 18 months, it is evident that emergence of pathology does not strictly correlate with manifestation of disease and raises the question as to when does CNS pathology become disease. The observation of pathology without a clinical disease phenotype can be a feature of human neurodegenerative disease. We note a report of an individual with a C9orf72 hexanucleotide repeat expansion mutation who underwent temporal lobe resection for epilepsy five years before the onset of FTD (Vatsavayai et al., 2016). The resected tissue harbored RNA foci, dipeptide repeat protein inclusions, and loss of nuclear TDP-43 but lacked TDP-43-positive protein inclusions. Upon postmortem examination 8 years after FTD symptom onset, abundant TDP-43 protein inclusions were detected. This case highlights the evolution of pathology that may progress for many years without disease manifestation, before the burden ultimately cannot be tolerated.

Due to the fact that the CHCHD10-R15L transgenic mice live for a relatively long time and die unexpectedly, it is difficult to attribute a precise cause of death. In examining the skeletal muscle and cardiac pathology of these mice, we observed further striking pathology characterized by protein inclusions comprising CHCHD10, p62, and/or ubiquitin. Of note, CHCHD10-WT (H) transgenic mice display a similar, but much milder, muscle pathology indicating that overexpression of transgenic human CHCHD10 protein can result in abnormal protein inclusions in muscle. Mutant CHCHD10 exacerbates the disruption to the myofibrillar network that will disturb normal function over time and likely contributes to premature death of CHCHD10-R15L transgenic mice alongside pathological CNS axonal swellings. Obvious degeneration of cardiomyocytes with protein inclusions are apparent in CHCHD10-R15L (M) and CHCHD10-R15L (H) mice that may contribute to cardiac failure later in life. Indeed, echocardiographic analysis of an older cohort of mice revealed severe functional deficits in a 16-month-old CHCHD10-R15L (H) mouse 10 days prior to death. The wide time frame of age of death made it difficult to capture this in other mice of the same genotype and revealed the onset of echocardiographic deficits to be abrupt. Cardiac dysfunction has been reported in ALS patients and sometimes attributed as a cause of death (Rosenbohm et al., 2017). Cardiac dysfunction has also been reported in mouse models of a distinct motor neuron disease, spinal muscular atrophy (Heier et al., 2010; Shababi et al., 2010). This provides further evidence that comorbidity of motor neuron disease and cardiac dysfunction may be relevant to disease course and phenotype. Our data warrant consideration to cardiac dysfunction being given to patients with a CHCHD10 p.R15L mutation. Additional evidence that mutant CHCHD10 impacts upon multiple tissues is provided by three independently reported knock-in mouse models of the CHCHD10 p.S59L mutation (Anderson et al., 2019; Genin et al., 2019; Liu et al., 2020b). This mutation was identified in patients with complex and varying phenotypes including myopathy, ataxia, cognitive impairment, motor neuron disease, and deafness, as well as a singleton ALS-FTD case (Bannwarth et al., 2014). The knock-in mouse models report pathology of the nervous system, skeletal muscle, and heart. Although the pathology we describe in our CHCHD10-R15L transgenic mouse models is unique, the common involvement of these three systems across different mouse models highlights the importance of CHCHD10 in those systems.

Longitudinal study of the behavior and pathology of Chchd10-KO mice was unremarkable. This is largely consistent with a previous report using a different global knockout strategy, which identified a mild ADP-stimulated respiration deficiency in skeletal muscle mitochondria as well as electron dense structures of unknown origin in the heart (Burstein et al., 2018). A conditional skeletal muscle Chchd10-KO mouse model has also been studied and displays some mild motor deficits, along with neuromuscular junction structural and electrophysiological deficits (Xiao et al., 2019). Combined with a lack of pathology or behavioral
phenotype in Chchd10-KO mice, the data presented initially suggested a toxic gain-of-function of CHCHD10 p.R15L in CNS, skeletal muscle, and heart resulting in severe pathology causing premature death in a copy-number correlated fashion. The recent report of Chchd10/Chchd2 double KO mice displaying a cardiac phenotype brings forth the possibility that mutant CHCHD10 might act through a toxic gain-of-function or a combination of loss-of-function of mutant CHCHD10 and a dominant negative mechanism of action on wild-type CHCHD10 and CHCHD2 to prevent compensation in the heart. This is distinct from the CNS pathology, which appears to arise as result of a toxic gain-of-function of CHCHD10 p.R15L, as it is absent from CHCHD10-WT transgenic mice and Chchd10-KO mice and not reported in Chchd10/Chchd2 double KO mice.

In conclusion, we present here transgenic mouse models that provide a valuable preclinical resource that can be used to interrogate mechanisms of CHCHD10 p.R15L-ALS. Our work underlines genetic pleiotropy as an important consideration in disease causation, as it greatly expands the clinical pathology to systems previously excluded from consideration in the natural history of diseases such as ALS.

Limitations of the study
Limitations of the technology prevented precise regulation of the level of expression of the transgene. We sought to address this limitation by developing and characterizing five independent transgenic mouse lines. Although the CHCHD10-WT lines do not exhibit precisely matched transgene expression levels to the CHCHD10-R15L (M) and CHCHD10-R15L (H) lines, these mouse models remain a valuable preclinical resource displaying numerous striking pathologies. It is possible that some aspects of the phenotype of these models arise due to transgene overexpression, rather than the mutation, or a combination of both. Importantly, although the severity of the CNS pathology positively correlates with transgene expression level, the data indicate that it arises due to the mutation because it is present in all three CHCHD10-R15L lines and absent from the CHCHD10-WT (H) line that has a higher transgene expression level than the CHCHD10-R15L (L) line.

Resource availability
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Teepu Siddique (t-siddique@northwestern.edu).

Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
The published article includes all datasets generated or analyzed during this study.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102061.

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AUTHOR CONTRIBUTIONS
Conceptualization, É.B.R., Y.C.M, H-X.D., and T.S.; Methodology, É.B.R., J.Y., N.M., S.D., Y.C.M., H-X.D., and T.S.; Investigation, É.B.R., J.Y., S.D., and Y.C.M; Resources, S.D., Y.C.M., and T.S.; Writing—Original Draft, É.B.R., Writing—Review and Editing, É.B.R., J.Y., S.D., Y.C.M, H-X.D., and T.S.; Supervision, T.S.; Funding Acquisition, T.S.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Early death of ALS-linked CHCHD10-R15L transgenic mice with central nervous system, skeletal muscle, and cardiac pathology

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Figure S1. Breeding & Genotyping Summary. Related to Figure 1.

(A) Number of litters weaned.
(B) Average litter size at weaning day P21. Data are represented as mean +/- SEM.
(C) Number of mice weaned at P21.
(D) Percentage of weaned mice that were transgenic.
Figure S2. Survival analysis and rotarod testing of CHCHD10 transgenic mice separated by gender. Related to Figures 1 and 2, and Tables S7-S18.

(A) Kaplan-Meier survival analysis of male mice of the indicated CHCHD10 founder lines. Death of a mouse for unknown reasons is considered a death event, as indicated by a drop in the line. Mice still living or death of a mouse due to intervention by the investigators are considered censored events, as indicated by tick marks along a line. Median survival for CHCHD10-WT (L) is 921 days, n=6 death events and n=34 censored events. Median survival for CHCHD10-WT (H) is 1003 days, n=3 death events and n=25 censored events. Gehan-Breslow-Wilcoxon test, p=0.3454 in comparison to CHCHD10-WT (L) line. Median survival for CHCHD10-R15L (L) is 1068 days, n=3 death events and 51 censored events. Gehan-Breslow-Wilcoxon test, p=0.0361 in comparison to
CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, \( p=0.3496 \) in comparison to CHCHD10-WT (H) line. Median survival for CHCHD10-R15L (M) is 691 days, \( n=11 \) death events and \( n=52 \) censored events. Gehan-Breslow-Wilcoxon test, \( p=0.0977 \) comparison to CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, \( p=0.1283 \) in comparison to CHCHD10-WT (H) line. Median survival for CHCHD10-R15L (H) is 484 days, \( n=12 \) death events and \( n=17 \) censored events. Gehan-Breslow-Wilcoxon test, \( p=0.0007 \) in comparison to CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, \( p=0.0043 \) in comparison to CHCHD10-WT (H) line.

(B) Kaplan-Meier survival analysis of female mice of the indicated CHCHD10 founder lines. Death of a mouse for unknown reasons is considered a death event, as indicated by a drop in the line. Mice still living or death of a mouse due to intervention by the investigators are considered censored events, as indicated by tick marks along a line. Median survival for CHCHD10-WT (L) is 902 days, \( n=6 \) death events and \( n=42 \) censored events. Median survival for CHCHD10-WT (H) is undefined as the mice are still being aged out. \( n=4 \) death event and \( n=26 \) censored events. Gehan-Breslow-Wilcoxon test, \( p=0.363 \) in comparison to CHCHD10-WT (L) line. Median survival for CHCHD10-R15L (L) is 887 days, \( n=8 \) death events and 32 censored events. Gehan-Breslow-Wilcoxon test, \( p=0.7128 \) in comparison to CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, \( p=0.3621 \) in comparison to CHCHD10-WT (H) line. Median survival for CHCHD10 R15L (H) is 746 days, \( n=13 \) death events and \( n=55 \) censored events. Gehan-Breslow-Wilcoxon test, \( p=0.2028 \) comparison to CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, \( p=0.026 \) in comparison to CHCHD10-WT (H) line. Median survival for CHCHD10 R15L (H) is 1010 days, \( n=9 \) death events and \( n=13 \) censored events. Gehan-Breslow-Wilcoxon test, \( p=0.005 \) in comparison to CHCHD10-WT (L) line. Gehan-Breslow-Wilcoxon test, \( p=0.0012 \) in comparison to CHCHD10-WT (H) line.

(C) Male mouse rotarod performance. Mice were tested on an accelerating rotarod task every 60 days beginning at 60 days of age until all CHCHD10-R15L (M) mice in the cohort died. The overlying numbers indicate the number of mice from each line used to calculate mean latency to fall at each time point. Data are represented as mean +/- SEM. Two way ANOVA with Holm-Šidák post hoc statistical analysis summarized in Table S13-S18.

(D) Female mouse rotarod performance. Mice were tested on an accelerating rotarod task every 60 days beginning at 60 days of age until all CHCHD10-R15L (M) mice in the cohort died. The overlying numbers indicate the number of mice from each line used to calculate mean latency to fall at each time point. Data are represented as mean +/- SEM. Two way ANOVA with Holm-Šidák post hoc statistical analysis summarized in Table S7-S12.
Figure S3. Atypical immunostaining profile of CHCHD10 R15L transgenic mice. Related to Figures 3 and 4. 
(A-E) Immunohistochemistry of 10 month old male CHCHD10-R15L (M) mouse spinal cord using different antibodies targeting CHCHD10, demonstrating the ability to visualize axonal pathology using antibodies targeting different epitopes of CHCHD10. (A) In-house designed antibody. (B) Abgent. (C) Proteintech. (D) Millipore. (E) Sigma. Black asterisks indicate the central canal. Black arrows indicate examples of pathological swellings. Scale bars = 100µm. 
(F) CHCHD10 protein schematic indicating N- and C-termini and CHCH domain. Overlying bars represent epitopes used to generate indicated antibodies. The Millipore antibody was generated using an unknown 79 amino acid immunogen.
Figure S4. CNS axonal pathology evident in CHCHD10-R15L transgenic mice. Related to Figures 3 and 4.

(A-C) Immunofluorescence of 10 month old CHCHD10-R15L (M) transgenic mice spinal cord gray matter using antibodies targeting CHCHD10 and SMI 310R, phosphorylated neurofilament heavy chain protein. Co-localization indicates that the CHCHD10-positive process harboring pathological swellings (white arrows) is also SMI 310R-positive. Scale bars = 25µm.

(D-F) Immunofluorescence of 10 month old CHCHD10-R15L (M) transgenic mice spinal cord gray matter using antibodies targeting CHCHD10 and NF-68, neurofilament light chain protein. Co-localization indicates a CHCHD10-positive process harboring pathological swellings (white arrow) is also SMI 310R-positive. Scale bars = 25µm.

(G-I) Immunofluorescence of 10 month old male CHCHD10-R15L (M) transgenic mouse spinal cord gray matter using antibodies targeting CHCHD10 and the mitochondrial protein, ATP synthase α. The white arrows in (H) indicate examples of ATP synthase α immunoreactive components of pathological axonal swellings. Scale bars = 25µm.

(J-L) Immunofluorescence of 10 month old male CHCHD10-R15L (M) transgenic mouse spinal cord gray matter using antibodies targeting CHCHD10 and mitochondrial protein complex IV, subunit VIb. The white arrows in (I) indicate examples of complex IV, subunit VIb immunoreactive components of pathological axonal swellings. Scale bars = 25µm.
| Time (Days) | Anterior Horn | Anterior Commissure |
|------------|---------------|---------------------|
| 30         | A             | B                   |
| 60         | C             | D                   |
| 120        | E             | F                   |
| 300        | G             | H                   |
| 600        | I             | J                   |
Figure S5. Progression of CHCHD10-R15L spinal cord pathology. Related to Figures 3 and 4. 
(A-J) Immunohistochemistry, using an antibody targeting CHCHD10, of CHCHD10-R15L (M) transgenic mouse spinal cord displaying the progression of pathology of the indicated regions of the spinal cord at the indicated timepoints. Scale bars = 100µm.
Figure S6. Transmission electron microscopy images of CHCHD10-R15L (M) spinal cord. Related to Figures 3 and 4.

(A) Black asterisks indicate pathological swellings in axons traversing the spinal cord white matter of a 10 month old male mouse. The white box indicates the region magnified in B.

(B) Two pathological swellings in close apposition are apparent. The blue box indicates the region magnified in C. The red box indicates the region magnified in D.

(C) White arrow indicates a relatively typical mitochondrial profile. Black arrows indicate examples of membranous components of the pathological swelling.

(D) Black arrows indicate examples of membranous components of the pathological swelling.

(E) A myelinated axon in the spinal cord gray matter of a 5 month old male mouse harboring at least one pathologic swelling (black asterisk) is apparent. A mitochondrion is observed apposed to the swelling (white arrow).

(F) A range of mitochondrial profiles are apparent in myelinated axons in the spinal cord gray matter of a 5 month old male mouse. Multiple abnormal, swollen mitochondria are observed, possibly in an early stage of development of the pathology (white arrows).
Figure S7. Axonal swellings present in spinal cord anterior horn cholinergic neurons. Related to Figures 3 and 4. (A-L) Immunofluorescence of 4 month old CHCHD10-R15L (M) transgenic mouse spinal cord anterior horn using antibodies targeting CHCHD10 and Choline Acetyl Transferase (ChAT). Co-localization indicates that the ChAT-positive neurons harbor pathological axonal swellings. White boxes indicate the regions magnified in the panels below. Scale bar sizes indicated in figure.
| CHCHD10-WT (L) | CHCHD10-WT (H) | CHCHD10-R15L (L) | CHCHD10-R15L (M) | CHCHD10-R15L (H) | CHCHD10-R15L (H) |
|-----------------|-----------------|-------------------|-------------------|-------------------|-------------------|
| **Pons**        | **Brainstem**   | **Cerebellum**    |                   |                   |                   |
| A               | B               | C                 |                   |                   |                   |
| D               | E               | F                 |                   |                   |                   |
| G               | H               | I                 |                   |                   |                   |
| J               | K               | L                 |                   |                   |                   |
| M               | N               | O                 |                   |                   |                   |
Figure S8. Immunohistochemistry of indicated brain regions using an antibody targeting CHCHD10. Related to Figures 3 and 4.

(A-C) 20 month old female CHCHD10-WT (L) transgenic mouse. Scale bar in (A) = 200µm. Scale bar in (B, C) = 50µm.

(D-F) 13 month old female CHCHD10-WT (H) transgenic mouse. Scale bar in (D) = 200µm. Scale bar in (E, F) = 50µm.

(G-I) 12 month old female CHCHD10-R15L (L) transgenic mouse. Black arrows indicated examples of pathological swellings. Scale bar in (G) = 200µm. Scale bar in (H, I) = 50µm.

(J-L) 20 month old female CHCHD10-R15L (M) transgenic mouse. Black arrows indicated examples of pathological swellings. Scale bar in (J) = 200µm. Scale bar in (K, L) = 50µm.

(M-O) 14 month old female CHCHD10-R15L (H) transgenic mouse. Black arrows indicated examples of pathological swellings. Scale bar in (M) = 200µm. Scale bar in (N, O) = 50µm.
Figure S9. Femoral nerve motor branch axon counts and area distribution and mitochondrial transport analysis in spinal cord primary neurons. Related to Figures 3 and 4, and Table S34.

(A) Representative toluidine blue stained 1µm semithin transverse section of the femoral nerve motor branch from a CHCHD10-WT (H) transgenic mouse. Scale bar = 50µm.

(B) Representative toluidine blue stained 1µm semithin transverse section of the femoral nerve motor branch from a CHCHD10-R15L (M) transgenic mouse. Scale bar = 50 µm.

(C) Quantitation of the number of axons for the indicated genotype. CHCHD10 WT (H) n=3 9 month old mice. CHCHD10 R15L (M) n=4 7 month old mice. Data are represented as mean +/- SEM. Unpaired t test, ns p>0.05.

(D) Quantitation of the percentage of axons of the total population for the indicated genotype binned by axonal area. CHCHD10 WT (H) n=3 9 month old mice. CHCHD10 R15L (M) n=4 7 month old mice. Data are represented as mean +/- SEM. Unpaired t test statistical analysis for each axon area is summarized in Table S34.

(E) Distance travelled by mitochondria in primary spinal cord neurons derived from E12.5 CHCHD10-R15L (M) mice or non-transgenic littermates. Data are represented as mean +/- SEM. Unpaired t test, ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001

(F) Percent of time mitochondria observed to be stationary, moving anterogradely or retrogradely. Data are represented as mean +/- SEM. Unpaired t test, ns p>0.05, ** p<0.01.

(G) Density of mitochondria in primary spinal cord neurons. Data are represented as mean +/- SEM. Unpaired t test, ns p>0.05.

(H) Length of mitochondria in primary spinal cord neurons. Data are represented as mean +/- SEM. Unpaired t test, * p<0.05.

(I) Basal mitochondrial oxygen consumption of primary spinal cord neurons derived from E12.5 CHCHD10-R15L (M) mice or non-transgenic littermates. Data are represented as mean +/- SEM. Unpaired t test, ns p>0.05.
Figure S10. Immunohistochemistry of spinal cord anterior horn using indicated antibodies. Related to Figures 3 and 4.

(A-C) 20 month old male CHCHD10-WT (L) transgenic mouse. Black box in A represents region magnified in B. Scale bars A, C = 100µm. Scale bar B = 50µm.

(D-F) 35 month old male CHCHD10-WT (H) transgenic mouse. Black box in D represents region magnified in E. Scale bars D, F = 100µm. Scale bar E = 50µm. Black arrows indicate examples of rare p62-positive puncta.

(G-I) 20 month old female CHCHD10-R15L (L) transgenic mouse. Black box in G represents region magnified in H. Scale bars G, I = 100µm. Scale bar H = 50µm. Black arrows indicate examples of rare p62-positive puncta.

(J-L) 20 month old female CHCHD10-R15L (M) transgenic mouse. Black box in J represents region magnified in K. Scale bars J, L = 100µm. Scale bar K = 50µm. Black arrows indicate examples of abundant p62-positive puncta.

(M-O) 14 month old female CHCHD10-R15L (H) transgenic mouse. Black box in M represents region magnified in N. Scale bars M, O = 100µm. Scale bar N = 50µm. Black arrows indicate examples of abundant p62-positive puncta.
Figure S11. Skeletal muscle myopathic features. Relate to Figure 5.  
(A-F) Hematoxylin and eosin staining of gastrocnemius (A-C) and diaphragm (D-F) of 14 month old female CHCHD10-WT (H) and 15 month old female CHCHD10-R15L (M) mice. Abundant myopathic features are apparent in the CHCHD10-R15L (M) transgenic mice including central nuclei, hematoxylin invasion, eosinophilic cores. Milder myopathic changes are apparent in CHCHD10-WT (H) skeletal muscle with occasional central nuclei. Scale bars (A, B) = 100µm. Scale bar (C) = 25µm.  
(G-J) Transmission electron microscopy images of quadriceps from a 10 month old male CHCHD10-R15L (M) mouse. White box in G indicates the region magnified in H. White box in H indicates the region magnified in I. White box in I indicates the region magnified in J. The myofibrillar network is disturbed by large electron-dense, possibly fibrous material of unknown composition or origin.
**A**

Exon

Chchd10

Knockout Allele (1.7kb)

**B**

H₂O  ++  +/-  -/-

Knockout Allele

Chchd10

**C**

Chchd10 mRNA Levels

**D**

C57BL/6J

Spinal Cord Ventral Horn

**E**

Chchd10-KO

**F**

Heart

**G**
Figure S12. Chchd10-Knockout mouse design and pathology. Related to Figure 8.

(A) Design strategy employed by the UC Davis knockout mouse project to knockout Chchd10.
(B) PCR amplification of indicated alleles demonstrating knockout of Chchd10 in homozygous condition.
(C) qRT-PCR result demonstrating the lack of Chchd10 mRNA transcript in Chchd10-KO mouse spinal cord. Gapdh is used as a reference. Relative expression levels represent the mean of three biological and three technical replicates. Data are represented as mean +/- confidence intervals. Unpaired t test of \(\Delta Ct\), ***p<0.001.
(D) Immunohistochemical staining of 10 month old male C57BL/6J transverse spinal cord using an antibody targeting CHCHD10 does not reveal any obvious pathology. Scale bar = 100µm.
(E) Immunohistochemical staining of 10 month old female Chchd10-KO transverse spinal cord using an antibody targeting CHCHD10 does not reveal any obvious pathology. Scale bar = 100µm.
(F) Immunohistochemical staining of 22 month old female C57BL/6J heart using an antibody targeting CHCHD10 does not reveal any obvious pathology. Scale bar = 100µm.
(G) Immunohistochemical staining of 25 month old male Chchd10-KO heart using an antibody targeting CHCHD10 does not reveal any obvious pathology. Scale bar = 100µm.
Two-way ANOVA | Ordinary
--- | ---
Alpha | 0.05

| Source of Variation | % of total variation | P value |
--- | --- | ---
Interaction | 21.4 | <0.0001 |
Age | 32.41 | <0.0001 |
Genotype | 11.23 | <0.0001 |

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
--- | --- | --- | --- | --- | ---
Interaction | 120869 | 13 | 9298 | F (13, 168) = 4.547 | P<0.0001 |
Age | 183027 | 13 | 14079 | F (13, 168) = 6.885 | P<0.0001 |
Genotype | 63399 | 1 | 63399 | F (1, 168) = 31.01 | P<0.0001 |
Residual | 343521 | 168 | 2045 | | |

Table S1. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic mouse lines. Related to Figure 2A, Table S2.

| Sidak's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
--- | --- | --- | ---
WT (L) - R15L (M) (Days) | | | |
60 | -21.4 | -84.23 to 41.44 | 0.9952 |
120 | -3.42 | -66.25 to 59.42 | >0.9999 |
180 | 12.33 | -50.5 to 75.17 | >0.9999 |
240 | -4.418 | -69.19 to 60.35 | >0.9999 |
300 | -14.25 | -79.02 to 50.52 | >0.9999 |
360 | -0.619 | -67.79 to 66.55 | >0.9999 |
420 | -3.413 | -70.59 to 63.76 | >0.9999 |
480 | -5.813 | -72.99 to 61.36 | >0.9999 |
540 | -8.333 | -84.32 to 67.66 | >0.9999 |
600 | 27.22 | -54.4 to 108.8 | 0.9961 |
660 | 102.5 | 20.86 to 184.1 | 0.0041 |
720 | 145.8 | 40.47 to 251.2 | 0.001 |
780 | 191.8 | 49.3 to 334.3 | 0.0015 |
840 | 198.4 | 49.39 to 347.4 | 0.0018 |

Table S2. Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic mouse lines for each timepoint. Related to Figure 2A, Table S1.
Two-way ANOVA

Ordinary

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 13.32                | 0.0063  |
| Age                 | 28.55                | <0.0001 |
| Genotype            | 4.164                | 0.0022  |

ANOVA table

| Source of Variation | SS (Type III) | DF | MS  | F (DFn, DFd) | P value |
|---------------------|--------------|----|-----|--------------|---------|
| Interaction         | 93318        | 13 | 7178| F (13, 157) = 2.374 | P=0.0063 |
| Age                 | 200058       | 13 | 15389| F (13, 157) = 5.089 | P<0.0001 |
| Genotype            | 29175        | 1  | 29175| F (1, 157) = 9.648 | P=0.0022 |
| Residual            | 474745       | 157| 3024|              |         |

Table S3. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic mouse lines. Related to Figure 2A, Table S4.

Sidak’s multiple comparisons test

| Time (min) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|------------|-----------------------------|--------------------|------------------|
| R15L (M) - WT (H) |
| 60         | 39.77                       | -36.72 to 116.3    | 0.8507           |
| 120        | 5.321                       | -71.17 to 81.81    | >0.9999          |
| 180        | 11.59                       | -64.90 to 88.08    | >0.9999          |
| 240        | 5.023                       | -73.82 to 83.86    | >0.9999          |
| 300        | 29.48                       | -49.37 to 108.3    | 0.9882           |
| 360        | 3.014                       | -78.75 to 84.78    | >0.9999          |
| 420        | 9.4                         | -72.37 to 91.17    | >0.9999          |
| 480        | -3.46                       | -90.19 to 83.27    | >0.9999          |
| 540        | 6.19                        | -88.82 to 101.2    | >0.9999          |
| 600        | 5.444                       | -96.25 to 107.1    | >0.9999          |
| 660        | -68.64                      | -170.3 to 33.06    | 0.499            |
| 720        | -117                        | -252.7 to 18.79    | 0.1553           |
| 780        | -170                        | -351.4 to 11.38    | 0.0855           |
| 840        | -183.5                      | -364.9 to -2.121   | 0.0451           |

Table S4. Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic mouse lines for each timepoint. Related to Figure 2A, Table S3.
Two-way ANOVA | Ordinary
---|---
Alpha | 0.05

| Source of Variation | % of total variation | P value |
---|---|---|
Interaction | 1.474 | 0.9975 |
Age | 5.433 | 0.5888 |
Genotype | 1.445 | 0.085 |

| ANOVA table | SS (Type III) | DF | MS | F (DFn, DFd) | P value |
---|---|---|---|---|---|
Interaction | 9510 | 13 | 731.5 | F (13, 191) = 0.2351 | P=0.9975 |
Age | 35064 | 13 | 2697 | F (13, 191) = 0.8670 | P=0.5888 |
Genotype | 9328 | 1 | 9328 | F (1, 191) = 2.999 | P=0.0850 |
Residual | 594194 | 191 | 3111 |

Table S5. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic mouse lines. Related to Figure 2A, Table S6.

| Sidak’s multiple comparisons test | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
---|---|---|---|
WT (L) - WT (H) | | | |
60 | 18.37 | -59.00 to 95.74 | >0.9999 |
120 | 1.901 | -75.47 to 79.27 | >0.9999 |
180 | 23.93 | -53.45 to 101.3 | >0.9999 |
240 | 0.6049 | -76.77 to 77.98 | >0.9999 |
300 | 15.22 | -62.15 to 92.59 | >0.9999 |
360 | 2.395 | -74.98 to 79.77 | >0.9999 |
420 | 5.988 | -71.38 to 83.36 | >0.9999 |
480 | -9.273 | -91.99 to 73.44 | >0.9999 |
540 | -2.143 | -87.09 to 82.80 | >0.9999 |
600 | 32.67 | -52.28 to 117.6 | >0.9999 |
660 | 33.85 | -51.10 to 118.8 | >0.9999 |
720 | 28.88 | -64.69 to 122.4 | >0.9999 |
780 | 21.77 | -81.11 to 124.6 | >0.9999 |
840 | 14.89 | -101.2 to 130.9 | >0.9999 |

Table S6. Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic mouse lines for each timepoint. Related to Figure 2A, Table S5.
Two-way ANOVA

| Source of Variation | % of total variation |
|---------------------|----------------------|
| Interaction         | 19.46                |
| Age                 | 36.87                |
| Genotype            | 17.99                |

ANOVA table

| Source of Variation | SS  | DF | MS  | F (DFn, DFd) | P value |
|---------------------|-----|----|-----|--------------|---------|
| Interaction         | 84734 | 13   | 6518 | F (13, 74) = 2.379 | P=0.0100 |
| Age                 | 160567 | 13   | 12351 | F (13, 74) = 4.509 | P<0.0001 |
| Genotype            | 78359   | 1     | 78359  | F (1, 74) = 28.6   | P<0.0001 |
| Residual            | 202715  | 74    | 2739   |               |         |

Table S7. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic female mice. Related to Figure S2D, Table S8.

Sidak’s multiple comparisons test

| WT (L) - R15L (M) (Days) | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
|--------------------------|------------|--------------------|-----------------|
| 60                       | 7.444      | -103.7 to 118.5    | >0.9999         |
| 120                      | 14.78      | -96.32 to 125.9    | >0.9999         |
| 180                      | 25.58      | -85.51 to 136.7    | >0.9999         |
| 240                      | 6.639      | -104.5 to 117.7    | >0.9999         |
| 300                      | -6.167     | -117.3 to 104.9    | >0.9999         |
| 360                      | 24.92      | -86.18 to 136      | >0.9999         |
| 420                      | 26.22      | -84.88 to 137.3    | 0.9999          |
| 480                      | 13.08      | -98.01 to 124.2    | >0.9999         |
| 540                      | 4.333      | -106.8 to 115.4    | >0.9999         |
| 600                      | 36.5       | -74.6 to 147.6     | 0.9961          |
| 660                      | 106.8      | -4.347 to 217.8    | 0.0695          |
| 720                      | 149.6      | 13.52 to 285.6     | 0.0206          |
| 780                      | 209.6      | 33.95 to 385.3     | 0.0085          |
| 840                      | 220.5      | 28.07 to 412.9     | 0.0134          |

Table S8. Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic female mice for each timepoint. Related to Figure S2D, Table S7.
## Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 11.31                | 0.3397  |
| Age                 | 34.36                | 0.0003  |
| Genotype            | 1.423                | 0.1759  |

### ANOVA table

| Source of Variation | SS (Type III) | DF | MS    | F (DFn, DFd) | P value |
|---------------------|---------------|----|-------|--------------|---------|
| Interaction         | 60761         | 13 | 4674  | F (13, 79) = 1.140 | P=0.3397 |
| Age                 | 184570        | 13 | 14198 | F (13, 79) = 3.463 | P=0.0003 |
| Genotype            | 7647          | 1  | 7647  | F (1, 79) = 1.865 | P=0.1759 |
| Residual            | 323930        | 79 | 4100  |              |         |

**Table S9.** Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic female mice. Related to Figure S2D, Table S10.

### Sidak's multiple comparisons test

| Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---------------------------|--------------------|------------------|
| R15L (M) - WT (H)         |                    |                  |
| 60                        | 46.26              | -82.42 to 174.9  | 0.9908 |
| 120                       | 18.05              | -110.6 to 146.7  | >0.9999 |
| 180                       | 17.75              | -110.9 to 146.4  | >0.9999 |
| 240                       | 17.11              | -111.6 to 145.8  | >0.9999 |
| 300                       | 39.26              | -89.42 to 167.9  | 0.9982 |
| 360                       | 7.383              | -121.3 to 136.1  | >0.9999 |
| 420                       | -0.65              | -129.3 to 128.0  | >0.9999 |
| 480                       | 11.24              | -117.4 to 139.9  | >0.9999 |
| 540                       | 25.67              | -110.0 to 161.3  | >0.9999 |
| 600                       | 11.64              | -124.0 to 147.3  | >0.9999 |
| 660                       | -64.58             | -200.2 to 71.06  | 0.9095 |
| 720                       | -97.54             | -272.6 to 77.57  | 0.7682 |
| 780                       | -135.3             | -370.3 to 99.60  | 0.726  |
| 840                       | -157.6             | -392.5 to 77.32  | 0.4969 |

**Table S10.** Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic female mice for each timepoint. Related to Figure S2D, Table S9.
Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 1.225                | >0.9999 |
| Age                 | 9.795                | 0.6676  |
| Genotype            | 10.36                | 0.0014  |

ANOVA table

|          | SS (Type III) | DF | MS   | F (DFn, DFd)     | P value  |
|----------|---------------|----|------|------------------|----------|
| Interaction | 5244          | 13 | 403.4| F (13, 85) = 0.09893 | P > 0.9999 |
| Age       | 41930         | 13 | 3225 | F (13, 85) = 0.7910  | P = 0.6676 |
| Genotype  | 44336         | 1  | 44336| F (1, 85) = 10.87   | P = 0.0014 |
| Residual  | 346611        | 85 | 4078 |                  |          |

Table S11. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic female mice. Related to Figure S2D, Table S12.

| Sidak's multiple comparisons test | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|----------------------------------|---------------------------|-------------------|-----------------|
| WT (L) - WT (H)                  |                           |                   |                 |
| 60                               | 53.7                      | -73.62 to 181.0    | 0.9565          |
| 120                              | 32.83                     | -94.49 to 160.1    | 0.9995          |
| 180                              | 43.33                     | -83.99 to 170.7    | 0.9928          |
| 240                              | 23.74                     | -103.6 to 151.1    | >0.9999         |
| 300                              | 33.09                     | -94.23 to 160.4    | 0.9995          |
| 360                              | 32.3                      | -95.02 to 159.6    | 0.9996          |
| 420                              | 25.57                     | -101.7 to 152.9    | >0.9999         |
| 480                              | 33.33                     | -100.9 to 167.5    | 0.9997          |
| 540                              | 25.67                     | -108.5 to 159.9    | >0.9999         |
| 600                              | 48.14                     | -86.07 to 182.3    | 0.9884          |
| 660                              | 42.17                     | -92.04 to 176.4    | 0.9966          |
| 720                              | 52.05                     | -92.91 to 197.0    | 0.9883          |
| 780                              | 74.28                     | -90.09 to 238.6    | 0.9285          |

Table S12. Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic female mice for each timepoint. Related to Figure S2D, Table S11.
### Table S13. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic male mice. Related to Figure S2C, Table S14.

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 3.531                | 0.9239  |
| Age                 | 19.58                | 0.0458  |
| Genotype            | 5.125                | 0.0385  |

#### ANOVA table

| Source of Variation | SS      | DF  | MS  | F (DFn, DFd)   | P value |
|---------------------|---------|-----|-----|----------------|---------|
| Interaction         | 3523    | 8   | 440.4 | F (8, 59) = 0.386 | P=0.9239 |
| Age                 | 19532   | 8   | 2442 | F (8, 59) = 2.14  | P=0.0458 |
| Genotype            | 5113    | 1   | 5113 | F (1, 59) = 4.483 | P=0.0385 |
| Residual            | 67303   | 59  | 1141 |                |         |

Table S14. Sidak's multiple comparisons test of rotarod performance between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic male mice for each timepoint. Related to Figure S2C, Table S13.

| WT (L) - R15L (M) (Days) | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
|--------------------------|-------------|-------------------|------------------|
| 60                       | -44.47      | -105.8 to 16.85   | 0.3185           |
| 120                      | -17.98      | -79.29 to 43.34   | 0.9904           |
| 180                      | 1.733       | -59.58 to 63.05   | >0.9999          |
| 240                      | -10.67      | -75.7 to 54.37    | 0.9999           |
| 300                      | -19.83      | -84.86 to 45.21   | 0.9874           |
| 360                      | -22.19      | -92.99 to 48.61   | 0.9848           |
| 420                      | -31.92      | -102.7 to 38.88   | 0.8668           |
| 480                      | -13.44      | -84.24 to 57.36   | 0.9997           |
| 540                      | -0.3333     | -108.7 to 108.1   | >0.9999          |
Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 5.566                | 0.9201  |
| Age                 | 6.859                | 0.8629  |
| Genotype            | 0.001808             | 0.9747  |

ANOVA table

| Source of Variation | SS (Type III) | DF | MS  | F (DFn, DFd)       | P value |
|---------------------|---------------|----|-----|--------------------|---------|
| Interaction         | 7097          | 8  | 887.1 | F (8, 49) = 0.3912 | P=0.9201 |
| Age                 | 8746          | 8  | 1093 | F (8, 49) = 0.4821 | P=0.8629 |
| Genotype            | 2.306         | 1  | 2.306| F (1, 49) = 0.001017| P=0.9747 |
| Residual            | 111115        | 49 | 2268 |

Table S15. Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic male mice. Related to Figure S2C, Table S16.

Didak's multiple comparisons test

| R15L (M) - WT (H) (Days) | Predicted (LS) mean diff. | 95.00% CI of diff.       | Adjusted P Value |
|--------------------------|--------------------------|--------------------------|------------------|
| 60                       | 34.08                    | -58.33 to 126.5          | 0.9549           |
| 120                      | -3.578                   | -95.98 to 88.33          | >0.9999          |
| 180                      | 7.183                    | -85.22 to 99.59          | >0.9999          |
| 240                      | -6.833                   | -104.2 to 90.57          | >0.9999          |
| 300                      | 18.36                    | -79.04 to 115.8          | 0.9997           |
| 360                      | -2.269                   | -107.5 to 102.9          | >0.9999          |
| 420                      | 22.71                    | -82.50 to 127.9          | 0.999            |
| 480                      | -35.07                   | -147.5 to 77.40          | 0.9847           |
| 540                      | -38.15                   | -197.2 to 120.9          | 0.9977           |

Table S16. Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic male mice for each timepoint. Related to Figure S2C, Table S15.
### Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 3.241                | 0.9678  |
| Age                 | 2.92                 | 0.9722  |
| Genotype            | 3.952                | 0.1324  |

### ANOVA table

| Source of Variation | SS (Type III) | DF | MS       | F (DFn, DFd)       | P value |
|---------------------|---------------|----|----------|--------------------|---------|
| Interaction         | 5085          | 8  | 676.9    | F (8, 59) = 0.2869 | P=0.9678|
| Age                 | 4582          | 8  | 644.7    | F (8, 59) = 0.2733 | P=0.9722|
| Genotype            | 6201          | 1  | 5492     | F (1, 59) = 2.328  | P=0.1324|
| Residual            | 139987        | 59 | 2359     |                    |         |

**Table S17.** Two-way ANOVA analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic male mice. Related to Figure S2C, Table S18.

### Sidak’s multiple comparisons test

| WT (L) - WT (H) (Days) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|------------------------|---------------------------|--------------------|------------------|
| 60                     | -10.39                    | -103.3 to 82.56    | >0.9999          |
| 120                    | -21.56                    | -114.5 to 71.39    | 0.9983           |
| 180                    | 8.917                     | -84.03 to 101.9    | >0.9999          |
| 240                    | -17.5                     | -110.4 to 75.45    | 0.9997           |
| 300                    | -1.467                    | -94.42 to 91.48    | >0.9999          |
| 360                    | -24.46                    | -117.4 to 68.49    | 0.9956           |
| 420                    | -9.206                    | -102.2 to 83.74    | >0.9999          |
| 480                    | -48.52                    | -149.7 to 52.67    | 0.8212           |
| 540                    | -38.48                    | -144.3 to 67.35    | 0.9602           |

**Table S18.** Holm-Šidák post hoc statistical analysis of rotarod performance between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic male mice for each timepoint. Related to Figure S2C, Table S17.
| Two-way ANOVA | Ordinary |
|---------------|----------|
| Alpha         | 0.05     |

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 15.81                | 0.0081  |
| Age                 | 27.37                | <0.0001 |
| Genotype            | 21.25                | <0.0001 |

| ANOVA table | SS  | DF  | MS       | F (DFn, DFd) | P value |
|-------------|-----|-----|----------|--------------|---------|
| Interaction | 649 | 13  | 49.93    | F (13, 74) = 2.449 | P=0.0081 |
| Age         | 1124| 13  | 86.42    | F (13, 74) = 4.24 | P<0.0001 |
| Genotype    | 872.2| 1   | 872.2    | F (1, 74) = 42.79 | P<0.0001 |
| Residual    | 1508| 74  | 20.38    |              |         |

Table S19. Two-way ANOVA analysis of body weight between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic female mice. Related to Figure 2C, Table S20.

| Sidak's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
|----------------------------------|------------|--------------------|------------------|
| WT (L) - R15L (M) (Days)         |            |                    |                  |
| 60                               | -1.25      | -10.83 to 8.333    | >0.9999          |
| 120                              | -2.5       | -12.08 to 7.083    | 0.9997           |
| 180                              | -0.125     | -9.708 to 9.458    | >0.9999          |
| 240                              | 2.775      | -6.808 to 12.36    | 0.999            |
| 300                              | 4.175      | -5.408 to 13.76    | 0.952            |
| 360                              | 3.925      | -5.658 to 13.51    | 0.9707           |
| 420                              | 6.225      | -3.358 to 15.81    | 0.5469           |
| 480                              | 9.85       | 0.2667 to 19.43    | 0.0393           |
| 540                              | 11.78      | 2.192 to 21.36     | 0.006            |
| 600                              | 11.85      | 2.267 to 21.43     | 0.0055           |
| 660                              | 12.18      | 2.592 to 21.76     | 0.0039           |
| 720                              | 10.63      | -1.112 to 22.36    | 0.1087           |
| 780                              | 8.55       | -6.603 to 23.7     | 0.7509           |
| 840                              | 10.55      | -6.049 to 27.15    | 0.5812           |

Table S20. Holm-Šidák post hoc statistical analysis of body weight between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic female mice for each timepoint. Related to Figure 2C, Table S19.
### Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 13.3                 | 0.0268  |
| Age                 | 44.77                | <0.0001 |
| Genotype            | 1.227                | 0.132   |

#### ANOVA table

| Source of Variation | SS (Type III) | DF | MS  | F (DFn, DFd) | P value |
|---------------------|---------------|----|-----|--------------|---------|
| Interaction         | 116.7         | 12 | 9.722 | F (12, 77) = 2.095 | P=0.0268 |
| Age                 | 392.7         | 12 | 32.72| F (12, 77) = 7.051 | P<0.0001 |
| Genotype            | 10.76         | 1  | 10.76| F (1, 77) = 2.318  | P=0.1320 |
| Residual            | 357.4         | 77 | 4.641|              |         |

**Table S21.** Two-way ANOVA analysis of body weight between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic female mice. Related to Figure 2C, Table S22.

### Sidak's multiple comparisons test

| R15L (M) - WT (H) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|-------------------|---------------------------|--------------------|-----------------|
| 60                | 0.93                      | -3.366 to 5.226    | >0.9999         |
| 120               | 2.11                      | -2.186 to 6.406    | 0.876           |
| 180               | 1.815                     | -2.481 to 6.111    | 0.9555          |
| 240               | 2.645                     | -1.651 to 6.941    | 0.6165          |
| 300               | 3.035                     | -1.261 to 7.331    | 0.4037          |
| 360               | 4.32                      | 0.02395 to 8.616   | 0.0477          |
| 420               | 1.9                       | -2.396 to 6.196    | 0.9379          |
| 480               | 0.725                     | -3.803 to 5.253    | >0.9999         |
| 540               | -1.375                    | -5.903 to 3.153    | 0.9975          |
| 600               | -1.1                      | -5.628 to 3.428    | 0.9998          |
| 660               | -3.4                      | -7.928 to 1.128    | 0.3135          |
| 720               | -1.883                    | -7.730 to 3.963    | 0.9956          |
| 780               | -0.75                     | -8.593 to 7.093    | >0.9999         |

**Table S22.** Holm-Šidák post hoc statistical analysis of body weight between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic female mice for each timepoint. Related to Figure 2C, Table S21.
### Two-way ANOVA

| Source of Variation | % of total variation | P value   |
|---------------------|----------------------|-----------|
| Interaction         | 9.509                | 0.0422    |
| Age                 | 30.45                | <0.0001   |
| Genotype            | 27.93                | <0.0001   |

### ANOVA table

| Source of Variation | SS (Type III) | DF | MS  | F (DFn, DFd)     | P value   |
|---------------------|---------------|----|-----|------------------|-----------|
| Interaction         | 397.6         | 12 | 33.13 | F (12, 82) = 1.930 | P=0.0422  |
| Age                 | 1273          | 12 | 106.1 | F (12, 82) = 6.183 | P<0.0001  |
| Genotype            | 1168          | 1  | 1168  | F (1, 82) = 68.06 | P<0.0001  |
| Residual            | 1407          | 82 | 17.16 |                   |           |

Table S23. Two-way ANOVA analysis of body weight between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic female mice. Related to Figure 2C, Table S24.

### Sidak's multiple comparisons test

| WT (L) - WT (H) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|-----------------|----------------------------|--------------------|------------------|
| 60              | -0.32                      | -8.566 to 7.926    | >0.9999          |
| 120             | -0.39                      | -8.636 to 7.856    | >0.9999          |
| 180             | 1.69                       | -6.556 to 9.936    | >0.9999          |
| 240             | 5.42                       | -2.826 to 13.67    | 0.5178           |
| 300             | 7.21                       | -1.036 to 15.46    | 0.1365           |
| 360             | 8.245                      | -0.0009181 to 16.49| 0.05             |
| 420             | 8.125                      | -0.1209 to 16.37   | 0.0566           |
| 480             | 10.58                      | 1.883 to 19.27     | 0.0068           |
| 540             | 10.4                       | 1.708 to 19.09     | 0.0083           |
| 600             | 10.75                      | 2.058 to 19.44     | 0.0056           |
| 660             | 8.775                      | 0.08304 to 17.47   | 0.0461           |
| 720             | 8.742                      | -0.6467 to 18.13   | 0.0882           |
| 780             | 7.8                        | -2.845 to 18.45    | 0.3499           |

Table S24. Holm-Šidák post hoc statistical analysis of body weight between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic female mice for each timepoint. Related to Figure 2C, Table S23.
Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 26.74                | <0.0001 |
| Age                 | 24.39                | <0.0001 |
| Genotype            | 20.47                | <0.0001 |

### ANOVA table

| Source of Variation | SS   | DF | MS   | F (DFn, DFd)   | P value |
|---------------------|------|----|------|----------------|---------|
| Interaction         | 727  | 8  | 90.88| F (8, 59) = 9.352 | P<0.0001|
| Age                 | 663.1| 8  | 82.88| F (8, 59) = 8.529 | P<0.0001|
| Genotype            | 556.4| 1  | 556.4| F (1, 59) = 57.26 | P<0.0001|
| Residual            | 573.4| 59 | 9.718|                 |         |

**Table S25.** Two-way ANOVA analysis of body weight between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic male mice. Related to Figure 2B, Table S26.

### Sidak’s multiple comparisons test

| WT (L) - R15L (M) (Days) | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
|---------------------------|------------|--------------------|------------------|
| 60                        | -1.64      | -7.299 to 4.019    | 0.9912           |
| 120                       | -3.02      | -8.679 to 2.639    | 0.7172           |
| 180                       | -1.66      | -7.319 to 3.999    | 0.9904           |
| 240                       | 4.39       | -1.613 to 10.39    | 0.308            |
| 300                       | 6.855      | 0.8524 to 12.86    | 0.0157           |
| 360                       | 8.673      | 2.139 to 15.21     | 0.003            |
| 420                       | 10.64      | 4.105 to 17.17     | 0.0002           |
| 480                       | 15.49      | 8.959 to 22.03     | <0.0001          |
| 540                       | 12.75      | 2.746 to 22.75     | 0.0049           |

**Table S26.** Holm-Šidák post hoc statistical analysis of body weight between CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic male mice for each timepoint. Related to Figure 2B, Table S25.
Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 28.48                | <0.0001 |
| Age                 | 37.55                | <0.0001 |
| Genotype            | 3.193                | 0.022   |

ANOVA table

| Source of Variation | SS (Type III) | DF | MS | F (DFn, DFd) | P value |
|---------------------|---------------|----|----|--------------|---------|
| Interaction         | 119.5         | 8  | 14.93 | F (8, 49) = 6.238 | P<0.0001 |
| Age                 | 157.6         | 8  | 19.7  | F (8, 49) = 8.226 | P<0.0001 |
| Genotype            | 13.4          | 1  | 13.4  | F (1, 49) = 5.595 | P=0.0220 |

Residual 117.3 49 2.394

Table S27. Two-way ANOVA analysis of body weight between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic male mice. Related to Figure 2B, Table S28.

Sidak’s multiple comparisons test

| R15L (M) - WT (H) (Days) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|--------------------------|----------------------------|--------------------|------------------|
| 60                       | 0.475                      | -2.528 to 3.478    | >0.9999          |
| 120                      | 7.56                       | 4.557 to 10.56     | <0.0001          |
| 180                      | 1.645                      | -1.358 to 4.648    | 0.6817           |
| 240                      | 1.05                       | -2.115 to 4.215    | 0.9769           |
| 300                      | 0.8                        | -2.365 to 3.965    | 0.9966           |
| 360                      | 1.192                      | -2.227 to 4.610    | 0.9682           |
| 420                      | -0.825                     | -4.244 to 2.594    | 0.9976           |
| 480                      | -2.133                     | -5.788 to 1.521    | 0.6034           |
| 540                      | -1.167                     | -6.335 to 4.002    | 0.9986           |

Table S28. Holm-Šidák post hoc statistical analysis of body weight between CHCHD10-WT (H) and CHCHD10-R15L (M) transgenic male mice for each timepoint. Related to Figure 2B, Table S27.
| Two-way ANOVA | Ordinary |
|---------------|----------|
| Alpha         | 0.05     |

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 13.9                 | <0.0001 |
| Age                 | 32.07                | <0.0001 |
| Genotype            | 28.87                | <0.0001 |

| ANOVA table | SS (Type III) | DF  | MS   | F (DFn, DFd)       | P value |
|-------------|---------------|-----|------|-------------------|---------|
| Interaction | 421           | 8   | 52.62| F (8, 60) = 5.353 | P<0.0001|
| Age         | 971.6         | 8   | 121.4| F (8, 60) = 12.35 | P<0.0001|
| Genotype    | 874.4         | 1   | 874.4| F (1, 60) = 88.95 | P<0.0001|
| Residual    | 589.8         | 60  | 9.83 |                   |         |

Table S29. Two-way ANOVA analysis of body weight between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic male mice. Related to Figure 2B, Table S30.

| Sidak's multiple comparisons test | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|----------------------------------|---------------------------|--------------------|------------------|
| WT (L) - WT (H) (Days)           |                           |                    |                  |
| 60                               | -1.165                    | -7.198 to 4.868    | 0.9996           |
| 120                              | 4.54                      | -1.493 to 10.57    | 0.2736           |
| 180                              | -0.015                    | -6.048 to 6.018    | >0.9999          |
| 240                              | 5.44                      | -0.5933 to 11.47   | 0.1041           |
| 300                              | 7.655                     | 1.622 to 13.69     | 0.0051           |
| 360                              | 9.865                     | 3.832 to 15.90     | 0.0001           |
| 420                              | 9.815                     | 3.782 to 15.85     | 0.0002           |
| 480                              | 13.36                     | 6.792 to 19.93     | <0.0001          |
| 540                              | 11.58                     | 4.714 to 18.45     | <0.0001          |

Table S30. Holm–Šidák post hoc statistical analysis of body weight between CHCHD10-WT (L) and CHCHD10-WT (H) transgenic male mice for each timepoint. Related to Figure 2B, Table S29.
| Limb | Belt Speed (cm/s) | Stride (s) | Stride Length (cm) | Stride Frequency (steps/s) |
|------|------------------|------------|---------------------|---------------------------|
| Average WT (L) | Right Hind | 10 | 0.362 | 3.633 | 2.817 |
| SEM WT (L) | Right Hind | 0.010 | 0.088 | 0.070 |
| Average R15L (M) | Right Hind | 10 | 0.398 | 3.967 | 2.589 |
| SEM R15L (M) | Right Hind | 0.010 | 0.093 | 0.061 |

P-Value: WT (L) Vs R15L (M) | Right Hind | 0.027 | 0.028 | 0.031 |

| Limb | Belt Speed (cm/s) | Overlap Distance (cm) |
|------|------------------|------------------------|
| Average WT (L) | Left Fore | 17 | 1.613 |
| SEM WT (L) | Left Fore | 0.117 |
| Average R15L (M) | Left Fore | 17 | 1.157 |
| SEM R15L (M) | Left Fore | 0.141 |

P-Value: WT (L) Vs R15L (M) | Left Fore | 0.039 |

| Limb | Belt Speed (cm/s) | Stride (s) | Stride Length (cm) | Stride Frequency (steps/s) | Paw Angle (Degrees) | Absolute Paw Angle (Degrees) | #Steps | Paw Area Variability at Peak Stance (cm²) |
|------|------------------|------------|---------------------|---------------------------|---------------------|-----------------------------|--------|----------------------------------|
| Average WT (L) | Right Hind | 17 | 0.293 | 4.967 | 3.483 | 17.350 | 17.350 | 15.500 | 0.058 |
| SEM WT (L) | Right Hind | 0.007 | 0.131 | 0.079 | 1.325 | 1.325 | 1.325 | 0.428 | 0.009 |
| Average R15L (M) | Right Hind | 17 | 0.325 | 5.522 | 3.156 | 22.467 | 22.467 | 13.333 | 0.039 |
| SEM R15L (M) | Right Hind | 0.010 | 0.161 | 0.093 | 1.471 | 1.471 | 1.471 | 0.717 | 0.004 |

P-Value: WT (L) Vs R15L (M) | Right Hind | 0.029 | 0.029 | 0.027 | 0.031 | 0.031 | 0.041 | 0.044 |

| Limb | Belt Speed (cm/s) | Stance Width (cm) | MIN dA/dT (cm²/s) | Paw Angle (Degrees) | Absolute Paw Angle (Degrees) | Axis Distance (cm) |
|------|------------------|------------------|-----------------|---------------------|-----------------------------|-------------------|
| Average WT (L) | Left Hind | 24 | 2.180 | -11.840 | 16.060 | 16.060 | 1.204 |
| SEM WT (L) | Left Hind | 0.086 | 0.481 | 2.168 | 2.168 | 2.168 | 0.053 |
| Average R15L (M) | Left Hind | 24 | 2.425 | -9.283 | 21.663 | 21.663 | 1.315 |
## Table S31

Summary of statistically significant differences of indicated gait parameters between 120 day old CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic mice.

|                  | Left Hind |         |         |         |         |
|------------------|-----------|---------|---------|---------|---------|
| SEM R15L (M)     |           | 0.037   | 0.386   | 0.991   | 0.991   | 0.019   |
|                  |           |         |         |         |         |         |
| P-Value: WT (L)  | Left Hind | 0.012   | 0.002   | 0.022   | 0.022   | 0.040   |
| Vs R15L (M)      |           |         |         |         |         |         |
| Limb | Belt Speed (cm/s) | Midline Distance (cm) |            |            |            |
|------|------------------|-----------------------|------------|------------|------------|
| Average WT (L) | Right Hind | 10 | 2.244 |            |            |
| SEM WT (L) | Right Hind |        | 0.218 |            |            |
| Average R15L (M) | Right Hind | 10 | 1.659 |            |            |
| SEM R15L (M) | Right Hind |        | 0.080 |            |            |
| **P-Value: WT (L) Vs R15L (M)** | Right Hind |     | 0.010 |            |            |

| Limb | Belt Speed (cm/s) | Propel (s) | % Propel | % Brake | % Propel |
|------|------------------|------------|----------|---------|----------|
| Average WT (L) | Right Fore | 17 | 0.142 | 43.280 | 29.500 | 70.500 |
| SEM WT (L) | Right Fore |    | 0.008 | 2.540 | 4.966 | 4.966 |
| Average R15L (M) | Right Fore | 17 | 0.121 | 35.250 | 41.138 | 58.863 |
| SEM R15L (M) | Right Fore |    | 0.009 | 2.666 | 5.112 | 5.112 |
| **P-Value: WT (L) Vs R15L (M)** | Right Fore |    | 0.007 | 0.009 | 0.030 | 0.030 |

| Limb | Belt Speed (cm/s) | Axis Distance (cm) |            |            |            |
|------|------------------|-------------------|------------|------------|------------|
| Average WT (L) | Right Hind | 17 | 1.484 |            |            |
| SEM WT (L) | Right Hind |    | 0.083 |            |            |
| Average R15L (M) | Right Hind | 17 | 1.298 |            |            |
| SEM R15L (M) | Right Hind |    | 0.039 |            |            |
| **P-Value: WT (L) Vs R15L (M)** | Right Hind |    | 0.042 |            |            |

| Limb | Belt Speed (cm/s) | Stance Width CV (%) | MAX dA/dT (cm²/s) |            |            |
|------|------------------|---------------------|-------------------|------------|------------|
| Average WT (L) | Left Fore | 24 | 34.580 | 18.875 |            |
| SEM WT (L) | Left Fore |    | 1.993 | 2.564 |            |
| Average R15L (M) | Left Fore | 24 | 43.610 | 11.256 |            |
| SEM R15L (M) | Left Fore |    | 1.951 | 1.213 |            |
| **P-Value: WT (L) Vs R15L (M)** | Left Fore |    | 0.015 | 0.023 |            |

| Limb | Belt Speed (cm/s) | #Steps |            |            |
|------|------------------|-------|------------|------------|
| Average WT (L) | Right Fore | 24 | 22.875 |            |
| SEM WT (L) | Right Fore |    | 0.774 |            |
| Average R15L (M) | Right Fore | 24 | 18.100 |            |
| SEM R15L (M) | Right Fore |    | 1.478 |            |
| Limb         | Belt Speed (cm/s) | #Steps |
|--------------|------------------|--------|
| Average WT (L) | Left Hind    | 24     | 22.875 |
| SEM WT (L)   | Left Hind      |        | 0.875  |
| Average R15L (M) | Left Hind | 24     | 17.500 |
| SEM R15L (M) | Left Hind      |        | 1.483  |

| Limb         | Belt Speed (cm/s) | #Steps |
|--------------|------------------|--------|
| Average WT (L) | Right Hind    | 24     | 23.000 |
| SEM WT (L)   | Right Hind     |        | 0.816  |
| Average R15L (M) | Right Hind | 24     | 17.900 |
| SEM R15L (M) | Right Hind     |        | 1.065  |

Table S32. Summary of statistically significant differences of indicated gait parameters between 600 day old CHCHD10-WT (L) and CHCHD10-R15L (M) transgenic mice.

| Belt Speed (cm/s) | 4 Months |         | 20 Months |
|-------------------|----------|---------|-----------|
|                   | WT (L) (n) | R15L (M) (n) | WT (L) (n) | R15L (M) (n) |
| 10                | 6         | 9        | 5         | 9           |
| 17                | 6         | 9        | 5         | 8           |
| 24                | 5         | 8        | 4         | 5           |

Table S33. Summary of the number of mice (n) of the indicated age and genotype that successfully completed the Digigait task.
| Axon Area | P value | Mean WT (L) | Mean R15L (M) |
|-----------|---------|-------------|---------------|
| <0.005    | 0.993   | 18.090      | 18.050        |
| <0.010    | 0.217   | 18.940      | 16.510        |
| <0.020    | 0.083   | 11.860      | 14.830        |
| <0.030    | 0.720   | 11.200      | 10.570        |
| <0.040    | 0.336   | 7.590       | 10.040        |
| <0.050    | 0.172   | 5.616       | 7.860         |
| <0.060    | 0.399   | 5.052       | 6.567         |
| <0.070    | 0.401   | 6.201       | 4.709         |
| <0.080    | 0.402   | 7.246       | 5.535         |
| <0.090    | 0.686   | 4.565       | 3.620         |
| <0.100    | 0.373   | 2.249       | 4.393         |
| <0.110    | 0.303   | 1.034       | 2.348         |

**Table S34.** Summary of unpaired t-test statistical analysis of the percentage of axons of a particular caliber comprising the femoral nerve motor branch population. Related to Figure S9D.
### Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 1.909                | 0.9153  |
| Row Factor          | 50.34                | <0.0001 |
| Column Factor       | 11.48                | <0.0001 |

#### ANOVA table

|                | SS (Type III) | DF | MS   | F (DFn, DFd) | P value |
|----------------|---------------|----|------|--------------|---------|
| Interaction    | 42.07         | 11 | 3.824| F (11, 87) = 0.4728 | P=0.9153 |
| Row Factor     | 1109          | 11 | 100.9| F (11, 87) = 12.47 | P<0.0001 |
| Column Factor  | 253.0         | 1  | 253.0| F (1, 87) = 31.28 | P<0.0001 |
| Residual       | 703.7         | 87 | 8.089|               |         |

Table S35. Two-way ANOVA analysis of body weight between C57BL/6J and Chchd10-KO male mice. Related to Figure 8A, Table S36.

#### Sidak’s multiple comparisons test

| C57BL/6 - Chchd10-KO (Days) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|-----------------------------|----------------------------|--------------------|------------------|
| 60                          | 1.537                      | -3.351 to 6.425    | 0.9951           |
| 120                         | 1.277                      | -3.778 to 6.331    | 0.9994           |
| 180                         | 1.540                      | -3.739 to 6.819    | 0.9976           |
| 240                         | 3.280                      | -1.999 to 8.559    | 0.5903           |
| 300                         | 3.320                      | -1.959 to 8.599    | 0.5723           |
| 360                         | 4.280                      | -0.9994 to 9.559   | 0.2107           |
| 420                         | 2.960                      | -2.319 to 8.239    | 0.7303           |
| 480                         | 4.140                      | -1.139 to 9.419    | 0.2506           |
| 540                         | 5.000                      | -0.2794 to 10.28   | 0.0771           |
| 600                         | 3.760                      | -3.224 to 10.74    | 0.7775           |
| 660                         | 3.350                      | -3.634 to 10.33    | 0.8813           |
| 720                         | 5.300                      | -4.339 to 14.94    | 0.7536           |

Table S36. Holm-Šidak post hoc statistical analysis of body weight between C57BL/6J and Chchd10-KO male mice for each timepoint. Related to Figure 8A, Table S35.
## Two-way ANOVA

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 3.187                | 0.9628  |
| Row Factor          | 35.06                | <0.0001 |
| Column Factor       | 0.008149             | 0.9071  |

### ANOVA table

| Source of Variation | SS (Type III) | DF  | MS     | F (DFn, DFd) | P value |
|---------------------|---------------|-----|--------|--------------|---------|
| Interaction         | 107.2         | 13  | 8.244  | F (13, 103) = 0.4115 | P=0.9628 |
| Row Factor          | 1179          | 13  | 90.69  | F (13, 103) = 4.527 | P<0.0001 |
| Column Factor       | 0.2740        | 1   | 0.2740 | F (1, 103) = 0.01368 | P=0.9071 |
| Residual            | 2064          | 103 | 20.03  |              |         |

**Table S37.** Two-way ANOVA analysis of body weight between C57BL/6J and Chchd10-KO female mice. Related to Figure 8B, Table S38.

### Sidak’s multiple comparisons test

| C57BL/6 - Chchd10-KO (Days) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|-----------------------------|---------------------------|--------------------|------------------|
| 60                          | 0.09667                   | -7.965 to 8.158    | >0.9999          |
| 120                         | -0.5900                   | -8.652 to 7.472    | >0.9999          |
| 180                         | -0.9067                   | -8.968 to 7.155    | >0.9999          |
| 240                         | 2.530                     | -5.532 to 10.59    | 0.9977           |
| 300                         | 0.4067                    | -7.655 to 8.468    | >0.9999          |
| 360                         | 1.467                     | -6.595 to 9.528    | >0.9999          |
| 420                         | 2.213                     | -5.848 to 10.27    | 0.9995           |
| 480                         | 2.280                     | -6.140 to 10.70    | 0.9995           |
| 540                         | 1.880                     | -6.540 to 10.30    | >0.9999          |
| 600                         | 0.6200                    | -8.311 to 9.551    | >0.9999          |
| 660                         | -1.200                    | -10.92 to 8.523    | >0.9999          |
| 720                         | -1.900                    | -12.07 to 8.268    | >0.9999          |
| 780                         | -3.750                    | -15.28 to 7.780    | 0.9967           |
| 840                         | -4.500                    | -17.81 to 8.813    | 0.9952           |

**Table S38.** Holm-Šidák post hoc statistical analysis of body weight between C57BL/6J and Chchd10-KO female mice for each timepoint. Related to Figure 8B, Table S37.
Two-way ANOVA analysis of rotarod performance between C57Bl/6J and Chchd10-KO mice. Related to Figure 8D, Table S40.

Table S39. Two-way ANOVA analysis of rotarod performance between C57Bl/6J and Chchd10-KO mice. Related to Figure 8D, Table S40.

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 3.487                | 0.8152  |
| Row Factor          | 5.081                | 0.5140  |
| Column Factor       | 0.2418               | 0.4470  |

ANOVA table

| Source of Variation | SS (Type III) | DF | MS | F (DFn, DFd) | P value |
|---------------------|---------------|----|----|--------------|---------|
| Interaction         | 14287         | 13 | 1099| F (13, 217) = 0.6439 | P=0.8152 |
| Row Factor          | 20821         | 13 | 1602| F (13, 217) = 0.9384 | P=0.5140 |
| Column Factor       | 990.6         | 1  | 990.6| F (1, 217) = 0.5804 | P=0.4470 |
| Residual            | 370352        | 217| 1707|               |         |

Table S40. Holm-Šidák post hoc statistical analysis of rotarod performance between C57BL/6J and Chchd10-KO mice for each timepoint. Related to Figure 8D, Table S39.

| Sidak's multiple comparisons test | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|----------------------------------|----------------------------|-------------------|-----------------|
| C57BL/6 - Chchd10 KO/KO          |                           |                   |                 |
| 60                               | -16.00                     | -67.06 to 35.06   | 0.9980          |
| 120                              | 2.165                      | -49.81 to 54.14   | >0.9999         |
| 180                              | -15.46                     | -68.50 to 37.58   | 0.9991          |
| 240                              | -0.8131                    | -53.85 to 52.22   | >0.9999         |
| 300                              | 2.531                      | -50.51 to 55.57   | >0.9999         |
| 360                              | -15.90                     | -68.94 to 37.14   | 0.9987          |
| 420                              | 11.61                      | -41.43 to 64.64   | >0.9999         |
| 480                              | -19.49                     | -73.77 to 34.80   | 0.9922          |
| 540                              | -3.856                     | -58.14 to 50.43   | >0.9999         |
| 600                              | -23.60                     | -86.28 to 39.08   | 0.9878          |
| 660                              | -32.37                     | -98.85 to 34.12   | 0.9039          |
| 720                              | -18.60                     | -94.69 to 57.48   | 0.9999          |
| 780                              | -4.675                     | -102.0 to 92.65   | >0.9999         |
| 840                              | 69.06                      | -52.33 to 190.4   | 0.7568          |

Table S40. Holm-Šidák post hoc statistical analysis of rotarod performance between C57BL/6J and Chchd10-KO mice for each timepoint. Related to Figure 8D, Table S39.
Two-way ANOVA analysis of rotarod performance between C57BL/6J and Chchd10-KO female mice. Related to Figure 8F, Table S42.

| Source of Variation | % of total variation | P value |
|---------------------|----------------------|---------|
| Interaction         | 6.914                | 0.8206  |
| Row Factor          | 6.999                | 0.8137  |
| Column Factor       | 0.1115               | 0.7162  |

ANOVA table

| Source of Variation | SS (Type III) | DF | MS | F (DFn, DFd)       | P value |
|---------------------|---------------|----|----|-------------------|---------|
| Interaction         | 16924         | 13 | 1302| F (13, 103) = 0.6338 | P=0.8206 |
| Row Factor          | 17132         | 13 | 1318| F (13, 103) = 0.6416 | P=0.8137 |
| Column Factor       | 273.0         | 1  | 273.0| F (1, 103) = 0.1329 | P=0.7162 |
| Residual            | 211558        | 103| 2054|                   |         |

Table S41. Two-way ANOVA analysis of rotarod performance between C57BL/6J and Chchd10-KO female mice. Related to Figure 8F, Table S42.

| C57BL/6 - Chchd10 KO/KO (Days) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|--------------------------------|----------------------------|--------------------|------------------|
| 60                             | -31.69                     | -113.3 to 49.94    | 0.9825           |
| 120                            | -3.281                     | -84.91 to 78.34    | >0.9999          |
| 180                            | -12.37                     | -94.00 to 69.25    | >0.9999          |
| 240                            | -6.985                     | -88.61 to 74.64    | >0.9999          |
| 300                            | 25.02                      | -56.60 to 106.6    | 0.9982           |
| 360                            | -12.19                     | -93.81 to 69.44    | >0.9999          |
| 420                            | 27.81                      | -53.81 to 109.4    | 0.9948           |
| 480                            | -8.200                     | -93.46 to 77.06    | >0.9999          |
| 540                            | 7.844                      | -77.41 to 93.10    | >0.9999          |
| 600                            | -23.66                     | -114.1 to 66.77    | 0.9997           |
| 660                            | -32.02                     | -130.5 to 66.42    | 0.9967           |
| 720                            | -31.14                     | -134.1 to 71.82    | 0.9985           |
| 780                            | -10.92                     | -127.7 to 105.8    | >0.9999          |
| 840                            | 69.06                      | -65.75 to 203.9    | 0.8592           |

Table S42. Holm-Šidák post hoc statistical analysis of rotarod performance between C57BL/6J and Chchd10-KO female mice for each timepoint. Related to Figure 8F, Table S41.
| Source of Variation | % of total variation | P value | P value summary |
|---------------------|----------------------|---------|----------------|
| Interaction         | 3.208                | 0.9851  | ns             |
| Row Factor          | 6.707                | 0.8082  | ns             |
| Column Factor       | 2.552                | 0.1112  | ns             |

**ANOVA table**

| Source of Variation | SS (Type III) | DF | MS | F (DFn, DFd) | P value |
|---------------------|---------------|----|----|--------------|---------|
| Interaction         | 4858          | 11 | 441.6 | F (11, 87) = 0.2960 | P=0.9851 |
| Row Factor          | 10157         | 11 | 923.3 | F (11, 87) = 0.6188 | P=0.8082 |
| Column Factor       | 3864          | 1  | 3864  | F (1, 87) = 2.589 | P=0.1112 |
| Residual            | 129824        | 87 | 1492  |                |         |

**Table S43.** Two-way ANOVA analysis of rotarod performance between C57BL/6J and Chchd10-KO male mice. Related to Figure 8F, Table S44.

| C57BL/6 - Chchd10 KO/KO (Days) | Predicted (LS) mean diff. | 95.00% CI of diff. | Adjusted P Value |
|--------------------------------|---------------------------|--------------------|------------------|
| 60                             | -1.686                    | -68.07 to 64.70    | >0.9999          |
| 120                            | 7.611                     | -61.04 to 76.26    | >0.9999          |
| 180                            | -19.09                    | -90.79 to 52.62    | 0.9990           |
| 240                            | 8.356                     | -63.35 to 80.06    | >0.9999          |
| 300                            | -20.42                    | -92.13 to 51.28    | 0.9981           |
| 360                            | -18.56                    | -90.26 to 53.15    | 0.9992           |
| 420                            | -3.244                    | -74.95 to 68.46    | >0.9999          |
| 480                            | -30.78                    | -102.5 to 40.93    | 0.9419           |
| 540                            | -15.56                    | -87.26 to 56.15    | 0.9999           |
| 600                            | -28.54                    | -123.4 to 66.31    | 0.9967           |
| 660                            | -30.59                    | -125.4 to 64.27    | 0.9939           |
| 720                            | -2.815                    | -133.7 to 128.1    | >0.9999          |

**Table S44.** Holm-Šidák post hoc statistical analysis of rotarod performance between C57BL/6J and Chchd10-KO male mice for each timepoint. Related to Figure 8F, Table S43.
Transparent Methods

Construction of CHCHD10 Transgene and Development of Transgenic Mice

The 6.4kb CHCHD10 transgene was amplified in 3 fragments from a human BAC clone RP11 124F9. This transgene includes 3.4kb upstream of the transcription start site to ensure inclusion of the endogenous CHCHD10 promoter. A 2.8kb fragment was PCR amplified from the BAC clone using a KpnI-anchored primer (P1 5'-CATAGGTACCTTACCTCTCCAACCTGATAAG3') and a primer immediately downstream from a SacII restriction site (P2 5'-GGGACTTGGGGCCAGCTCAGAT3'). This PCR product was digested with KpnI and SacII (New England Biolabs, Inc.), agarose gel purified, and cloned into the pBluescript II SK(-) plasmid vector. A 1.3kb fragment was PCR amplified from the BAC clone using a primer that includes the SacII restriction site (P3 5'-GCCTCATATCCCGCCGGGACTTGTAAG3') and a primer immediately downstream from an Xhol restriction site (P4 5'-GGGACTTGGGGCCAGCTCAGAT3'). This PCR product was digested with SacII and Xhol (New England Biolabs, Inc.), agarose gel purified, and cloned into the pBluescript II SK(-) plasmid vector. A 2.3kb fragment was PCR amplified from the BAC clone using a primer that includes the Xhol restriction site (P5 5'-CCAGGATTATCTCGAGGCAACACAG3') and a primer that includes a BamHI restriction site (P6 5'-CAGAGTGCGTACCTCTCTGACAG3'). This PCR product was digested with Xhol and BamHI (New England Biolabs, Inc.), agarose gel purified, and cloned into the pBluescript II SK(-) plasmid vector. A c.44 G > T (p.R15L) mutation was introduced into the 1.3kb SacII/Xhol fragment by site-directed mutagenesis (QuickChange Multi Site-Directed Mutagenesis Kit, #200515, Agilent Technologies, Inc.) (P7 5'-TCCCAACCCGCAGCTCAGCCGGCCT3'; bold T indicates mutation site, bold A indicates a single nucleotide polymorphism, rs179468). The entire CHCHD10-WT and CHCHD10-R15L transgenes were assembled by ligation of the 2.8kb KpnI/SacII fragment, 1.3kb SacII/Xhol fragment and 2.3kb Xhol/BamHI fragment into the pBluescript II SK(-) plasmid vector. The transgenes were released from their respective plasmids by restriction digestion with KpnI and BamHI, agarose-gel purified, and used for microinjection into fertilized eggs derived from a zygote of a C57BL/6 x SJL cross. Transgenic mice were identified by PCR using a primer set (CHCHD10-P8 5'-CCAGGTTTGAACGCACTCCA3' and CHCHD10-P9 5'- AGCTATCTGGTGTAATTTT3'). The relative transgene copy number was estimated by PCR using transgene-specific primers (CHCHD10-P8 and CHCHD10-P9) and mouse beta-actin gene specific primers (Actb-P10, 5'-TGTCCAATGCGGACA3' and Actb-P11, 5'-ACCTGGGTGACATTTT3') in the same PCR system. Subsequent generations were backcrossed on to a C57BL/6J background. Two CHCHD10-WT and three CHCHD10-R15L founder lines were established and studied.

Development of Chchd10 Knockout Mice

Mouse embryonic stem cells (ESCs) harboring a Chchd10 knockout allele (Chchd10^{tm1[KOMP]Lco}) (Project ID: 13977) were acquired from the UC Davis Knockout Mouse Project Repository. This allele replaces 1725bp of Chchd10 (chr10:75,935,967-75,937,691) with a LacZ reporter and neomycin selection cassette. The ESCs were implanted into a female mouse and resulting chimeric mice were interbred with C57BL/6J mice and heterozygous knockout mice were identified by PCR using knockout allele-specific primers (Chchd10-KO-P12, 5'-CACCTGACTCTAAGGACGCTGCCGCT3' and Chchd10-KO-P13, 5'-CCGTACCAAGATCTGAGTTGCTGC3'). Homozygous knockout mice were obtained by interbreeding heterozygous knockout mice and identified by PCR using knockout allele-specific primers and mouse Chchd10-specific primers (Chchd10-P14, 5'-CTGCCAGGGCGGTCAGTTGAG3' and Chchd10-P15, 5'-CCAGTGAGCCCTCTGACT3').

Animal Care and Use

Animal-use protocols have been approved by the Institutional Animal Care and Use Committee of Northwestern University for this project. All experiments were carried out according to regulatory standards. The mice used in this study were maintained on a C57BL/6J background (The Jackson Laboratory, stock no. 000664) and were housed in pathogen-free conditions in microisolation cages in the barrier facilities of Northwestern University Center for Comparative Medicine.

qRT-PCR

Total RNA was isolated from mouse spinal cord using an RNeasy Lipid Tissue Mini Kit (74804, Qiagen). Reverse transcription was carried out using a BcaBEST™ RNA PCR Kit (RR023A, Takara Bio Inc.) and quantitative PCR was performed using iTaq™ Universal SYBR® Green Supermix (1725121, Bio-Rad Laboratories, Inc.) and a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Primers used in quantitative PCR include (Chchd10 qRT-PCR F-P16, 5'-CTCCTATGAGATCAACGATCTCC3' and Chchd10 qRT-PCR R-P17, 5'-AGCTCAGAGCTGATTGTATT-3'; Gapdh qRT-PCR F-P18, 5'-AACAGCAACTCCACTCTTC3' and Gapdh qRT-PCR R-P19, 5'-CCTGGTGCTTAGCCGTATT-3').

Mouse Behavioral Testing

CHCHD10-WT (H), CHCHD10-WT (L) and CHCHD10-R15L (M) mice were tested on a RotaRod task every 60 days beginning at 60 days of age. Each test involved a trial on 3 consecutive days with each trial consisting of 3 experiments separated by at least 5 minutes. The latency to fall was recorded for each mouse as the RotaRod (Ugo Basile) accelerated from 5rpm to 40rpm up to a maximum of 5 minutes. Results indicate the average latencies to fall of all
experiments per test. The body weight of this cohort of mice was measured every 60 days beginning at 60 days of age. Separate cohorts of 4 month or 14-20 month old mice were subjected to an open field task. Each mouse was placed in a novel open field environment for five minutes and their movements were video recorded. Limelight software (Coulbourn Instruments) was used to determine total distance travelled, velocity and duration of time spent in the periphery and center of the open field. Gait analysis was carried out on 4 month and 20 month old mice using a DigiGait (Mouse Specifics, Inc.). Mice were placed on a treadmill moving at 10cm/s, 17cm/s and 24cm/s and various parameters related to gait features were recorded and analyzed using the DigiGait software. The numbers and gender of mice used are indicated in the figures and/or figure legends.

**Immunohistochemistry and Confocal Microscopy**

Mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (Electron Microscopy Sciences). 6µm sections were cut from paraffin-embedded spinal cord and brain. The sections were deparaffinized with xylene and rehydrated with a descending series of diluted ethanol and water. Antigens in the sections were retrieved using 1X antigen decloaker, a citrate buffer heat retrieval solution, pH 6.0 (Biocare Medical), and a high-pressure decloaking chamber. For immunohistochemistry, endogenous peroxidase activity was blocked with 3% hydrogen peroxide (BioGenex). Non-specific background was blocked with 1% bovine serum albumin (BSA) (Sigma Aldrich). Primary antibodies were diluted in 1% BSA and applied to sections at 4°C overnight. Biotinylated goat anti-rabbit and anti-mouse IgG were used as the secondary antibodies (Biocare Medical). Immunoreactive signals were detected with peroxidase-conjugated streptavidin (BioGenex) using 3-amino-9-ethylcarbazole (BioGenex) as a chromogen. The slides were counterstained with hematoxylin and mounted with Aqua PolyMount (Polyscience).

Brightfield light microscopy imaging was performed using an Olympus AX70 microscope. For confocal microscopy, non-specific background was blocked with 1% BSA. Primary antibodies were diluted in 1% BSA and applied to sections at 4°C overnight. The appropriate secondary antibody, anti-rabbit, anti-mouse or anti-goat IgG, conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen) were diluted in 1% BSA for 1 hour at room temperature. The slides were mounted with ProLong™ Gold antifade reagent with or without DAPI (Invitrogen). Fluorescence was detected using a Nikon A1R+ Confocal Laser Microscope System.

**Antibodies**

We designed and synthesized an antibody which was raised in rabbit using a polypeptide of human CHCHD10 (amino acids 17-30: AAPSAHPPAHPPPSA) (Biosynthesis, Inc.) The antiserum was affinity-purified. Other antibodies used in this study include anti-CHCHD10 (AP16303a, Abgent, Inc.; 25671-1-AP, Proteintech Group, Inc.; MABN1524, Millipore Sigma; HPA003440, Sigma), anti-ubiquitin (10R-U101b, Fitzgerald Industries International), anti-SQSTM1/P62 (H00008878-M01, Abnova), anti-ATP Synthase α subunit (612517, BD Transduction), COX IV subunit Vb (A-21366, Molecular Probes), anti-Map2 (M1406, Sigma), anti-SMI 32 (801701, Biolegend), anti-SMI 310R (Covance), anti-NF-68 (N5139, Sigma), and anti-ChAT (AB144p, Millipore), anti-TDP-43 (10782-2-AP, Proteintech).

**Western Blotting**

Mouse forebrain lysate was prepared using RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Sodium Deoxycholate, 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific)). Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific). 15µg total protein was separated on NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo Scientific) by electrophoresis and blotted onto PVDF membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% milk in PBS with 0.1% Tween20® (Sigma-Aldrich). Anti-CHCHD10 (AP16303a, Abgent, Inc.) diluted in 5% BSA (Sigma-Aldrich) was applied to the membranes overnight at 4°C. Secondary goat anti-rabbit IgG (H+L)-HRP conjugate (1721019, Bio-Rad Laboratories, Inc.) was diluted in 5% milk in PBS with 0.1% Tween20 and applied for 1 hour at room temperature. Chemiluminescence upon application of Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) was detected using an Azure Biosystems c600 imaging system. The membranes were stripped using Restore™ Western Blot Stripping Buffer (Thermo Scientific) and re-probed with anti-GAPDH (NB300-324, Novus Biologicals) to serve as a loading control.

**Electron Microscopy**

Mice were transcardially perfused under deep anesthesia with fixative (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (Electron Microscopy Sciences)). Post-fixation in 1% OsO₄ in 0.1M phosphate buffer was followed by dehydration through an ethanol series and propylene oxide before embedding using an EMbed812 kit (Electron Microscopy Sciences). 1µm semithin sections were stained with toluidine blue. 100nm ultrathin sections were contrasted with uranyl acetate, lead nitrate and sodium citrate. Electron microscopy images were acquired using an FEI Tecnai Spirit G2 transmission electron microscope.

**Femoral Nerve Motor Branch Axon Counts**

Dissection and procedure was carried out as described (Burgess et al., 2010). Nerve tissue was processed and embedded as described for electron microscopy sample preparation. 1µm semithin sections were stained with toluidine
blue. Light microscopy images were acquired using an Olympus AX70 microscope and merged using Photoshop. Axons numbers and areas were counted and calculated using Fiji software.

**Mouse primary spinal cord neuron culture**

Primary neurons from mouse spinal cords were cultured in Neurobasal™ medium (Life Technologies) supplemented with B-27™ (Life Technologies). Spinal cords from E12.5 mouse embryos were dissected out and dissociated with 0.25% trypsin. After enriching motor neurons with Optiprep™ (Sigma) density gradient centrifugation and a BSA cushion, cells were seeded on glass coverslips coated with 20μg/ml poly-L-lysine (Sigma) and 8μg/ml laminin (Sigma) and grown in the presence of 50μg/ml BDNF, 50μg/ml CNTF, and 25μg/ml GDNF (PeproTech). Neurons were transfected with Lipofectamine™ 2000 (Life Technologies) following manufacturer’s instructions.

**Oxygen Consumption Rate Measurements in Primary Spinal Cord Neurons**

Primary spinal cord neuron mitochondrial oxygen consumption rates (OCR) were measured using a Seahorse XFe96 Analyzer (Agilent). Basal mitochondrial OCR was determined by subtracting the OCR following antimycin A (Sigma) and rotenone (Sigma) treatment from the baseline OCR. OCR was normalized to cell number quantified by DAPI staining.

**Live imaging and data analysis of mitochondria transport, density and fragmentation**

Time-lapse live imaging by confocal microscope was used to measure axonal mitochondrial transport, density, and fragmentation. After culturing for 5–7 days, primary spinal cord neurons were transfected with mitochondria targeting sequence-tagged DsRed (mito-DsRed). 48 hours after transfection, images were acquired using a Zeiss LSM 700 confocal microscope equipped with a 63X/NA 1.15 water LD C-Apochromat objective lens and a temperature (37°C) and CO₂ (5%) controlled stage. Images were captured every two seconds for a period of two minutes using Zen 2009 software. The 561nm laser intensity was set at 0.2mW to minimize damage, and pinholes were opened maximally to allow the entire thickness of the axon to be imaged. Axon fragments of 50-100μm in length located at least 50μm away from the cell body were selected for analysis. Custom-made Image J plug-ins were used to generate kymographs and analyze mitochondria motility (Pekkurnaz et al., 2014). Distance travelled was defined as the average distance travelled by each mitochondrion in one minute; Percent of Time was defined as the average of time spent mobile in each direction. Mitochondria that moved continuously in one direction were scored as 100% of Time in motion for that direction, while those that were entirely stationary or only moved in the opposite direction were scored as 0% Time in motion for that direction. Mitochondria length and density were measured by using the first frame of each time-lapse recording on selected axons, and analyzed with Imaris software (Bitplane). Mitochondria density was calculated by dividing the total length of mitochondria with the length of axon in the same view field.

**Skeletal Muscle and Heart Sample Preparation**

Quadriceps, gastrocnemius and diaphragm skeletal muscle was collected from deeply anesthetized mice and flash frozen in liquid nitrogen-cooled isopentane. 4μm sections were cut using a cryostat and stained with hematoxylin and eosin. Mouse hearts were immersed in 4% paraformaldehyde and paraffin-embedded. 6μm sections were cut using a microtome and deparaffinization was carried out as described above. Immunostaining of skeletal and cardiac muscle was carried out as described above.

**Skeletal Muscle Respirometry**

High-resolution respirometry was performed using an Oroboros O2K (Oroboros Instruments, Innsbruck, Austria) per established protocols (Kuznetsov et al., 2008, LaBarge et al., 2016). Briefly, tibialis anterior and soleus muscles were harvested immediately after euthanasia and preserved in preservation solution (BIOPS; 2.77mM CaK₂EGTA, 7.23mM K₂EGTA, 5.7mM Na₂ATP, 6.56mM MgCl₂, 20mM taurine, 15mM Na₂Phosphocreatine, 20mM imidazole, 0.5mM DTT, and 50mM MES). The muscles were mechanically separated in ice-cold BIOPS under a dissecting microscope to obtain replicates of around 2-3mg and permeabilized with 50 μg/ml saponin for 30 min followed by a 10 min wash in mitochondrial respiration media [MiR05; 0.5mM EGTA, 3mM MgCl₂, 60mM K-lactobionate, 20mM taurine, 10mM KH₂PO₄, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 110mM sucrose, 1g/L fatty acid-free bovine serum albumin]. All data were collected at 37°C in hyperoxygenated (200–400μM O₂) conditions in MiR05 to avoid limitations with oxygen diffusion. The substrate-uncoupler-inhibitor titrations (SUIT) respiration protocol used to test for maximal phosphorylation and electron transport chain capacity of complex-I and complex-II mediated respiration was: 0.5mM malate, 5mM pyruvate, 5mM ADP, 10mM glutamate, 10mM cytochrome c, 10mM succinate, followed by 0.5mM titrations carbonyl cyanide m-chloro phenyl hydrazine (CCCP), 0.5mM rotenone, and 2.5mM antimycin A. Any replicates that were higher than 10% following the addition of cytochrome c were considered to be over-permeabilized and those data were not used. The state of respiration after addition of glutamate is considered complex-I (+III+IV+ATP Synthase) mediated phosphorylation capacity, that after succinate is complex-I+II (+III+IV+ATP Synthase), i.e. maximal phosphorylation capacity and the state after addition of CCCP (uncoupler) is maximal electron transport chain capacity.
**Echocardiography**

Mice were anesthetized by inhalation of 1.5% isoflurane. Parasternal short axis views were collected using a Vevo® 3100 Imaging System and analyzed with Vevo® LAB software (FUJIFILM Visual Sonics). M-mode measurements were used to calculate the reported parameters.
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