TDP-43 and PINK1 mediate CHCHD10S59L mutation-induced defects in Drosophila and in vitro

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Mutations in coiled-coil-helix-coiled-helix domain containing 10 (CHCHD10) can cause amyotrophic lateral sclerosis and frontotemporal dementia (ALS-FTD). However, the underlying mechanisms are unclear. Here, we generate CHCHD10S59L-mutant Drosophila melanogaster and HeLa cell lines to model CHCHD10-associated ALS-FTD. The CHCHD10S59L mutation results in cell toxicity in several tissues and mitochondrial defects. CHCHD10S59L independently affects the TDP-43 and PINK1 pathways. CHCHD10S59L expression increases TDP-43 insolubility and mitochondrial translocation. Blocking TDP-43 mitochondrial translocation with a peptide inhibitor reduced CHCHD10S59L-mediated toxicity. While genetic and pharmacological modulation of PINK1 expression and activity of its substrates rescues and mitigates the CHCHD10S59L-induced phenotypes and mitochondrial defects, respectively, in both Drosophila and HeLa cells. Our findings suggest that CHCHD10S59L-induced TDP-43 mitochondrial translocation and chronic activation of PINK1-mediated pathways result in dominant toxicity, providing a mechanistic insight into the CHCHD10 mutations associated with ALS-FTD.
In 2014, Bannwarth et al. identified an S59L substitution in coiled-coil-helix-coiled-coil-helix domain containing 10 (CHCHD10) as the causes of a familial disease characterized by motor function defects, declined cognitive function, and myopathy. Subsequent analyses revealed that CHCHD10S59L is a genetic cause of amyotrophic lateral sclerosis with frontotemporal dementia (ALS-FTD)1. CHCHD10 encodes a functionally unknown, small protein comprising a putative N-terminal mitochondrial-targeting sequence and a C-terminal CHCHD domain. Identification of mutant CHCHD10 in ALS-FTD and related diseases and its predominant localization in mitochondria provide an opportunity to investigate the mitochondrial origin of neurodegenerative diseases.

Many additional CHCHD10 variants are now known to cause ALS, FTD, and other related degenerative diseases2,3. However, the pathogenicity and penetrance of some variants are debatable. Although CHCHD10R15L was identified in familial and sporadic cases of ALS, the existence of unaffected carriers in familial cases suggests incomplete penetrance4–7. The CHCHD10P34S variant occurs in sporadic ALS8,9, ALS-FTD10, Parkinson disease6, and Alzheimer’s disease6, and its overexpression causes mitochondrial defects31. However, its pathogenicity is not well supported by genetic evidence12,13. Although some molecular mechanisms for such overexpression-mediated cell toxicity of CHCHD10S59L and CHCHD10G66V were identified in mitochondrial myopathy and late-onset spinal muscular atrophy, Jokela type (SMAJ), or Charcot-Marie-Tooth disease type 2 (CMT2), respectively5,14,15, muscular atrophy, Jokela type (SMAJ), or Charcot-Marie-Tooth disease type 2 (CMT2) has been independently suggested as a cause of ALS-FTD and other related diseases and its predominant localization in mitochondria provide an opportunity to investigate the mitochondrial origin of neurodegenerative diseases.

The CHCHD10 mutations identified in familial diseases are dominantly inherited1,4,5,14. However, experimental evidence does not support that all disease-causing variants have the same mode of action. Bannwarth et al. showed that CHCHD10 expression in patient tissues is not affected and that overexpression of CHCHD10S59L causes mitochondrial defects similar to that in affected patients. This suggests that CHCHD10S59L is a dominant gain-of-function mutant. Woo et al. also confirmed such overexpression-mediated cell toxicity of CHCHD10S59L. However, CHCHD10S59L does not retain its wild-type (WT)-like activity, indicative of a dominant-negative mechanism. Furthermore, patient fibroblasts with either CHCHD10R15L or CHCHD10G66V exhibit reduced expression and protein instability, supporting a haploinsufficiency mechanism30,21. These data indicate that more detailed investigation is necessary to understand the disease-causing mechanism(s) of mutant CHCHD10 and suggest that CHCHD10 mutations have multiple modes of action.

Although some molecular mechanisms for CHCHD10-mediated cell toxicity are known, it is unclear how these mechanisms drive the disease phenotype and whether they can be controlled therapeutically. CHCHD10 interacts with components of the mitochondrial contact site and cristae organizing system (MICOS), and the MICOS complex is decreased in patients with CHCHD10 mutations11. However, Straub et al. reported that CHCHD10 is not well localized with the MICOS complex and that CHCHD10–CHCHD2 hetero-complex formation decreases in patient fibroblasts carrying CHCHD10R15L. Although Woo et al. reported the physical interaction of CHCHD10 and TDP-43, Genin et al. demonstrated that phosphorylated TDP-43 levels are not associated with the phenotypic severity of CHCHD10S59L or CHCHD10G66V. Indeed, severity was more closely associated with MICOS complex formation22. Because of a lack of a genetically tractable model, most studies rely on the analysis of protein–protein interactions. Therefore, systematic evaluation of which upstream effectors or signaling pathways are essential for disease pathogenesis is lacking.

Identification of CHCHD10 mutations and pathogenic mitochondrial pathways in ALS-FTD suggest that mitochondrial defects are a primary cause of ALS, FTD, or other related diseases23–28. However, this also raises several intriguing questions. Are mitochondrial defects the primary driver of disease in specific subtypes of ALS-FTD? Do all of these subtypes share a common mechanism? Can mitochondrial defects in different subtypes be rescued by activating protective pathways or targeting a common pathogenic mechanism? To address these questions, we used Drosophila and mammalian cell culture models of CHCHD10S59L-mediated cell toxicity. In these models, we found that CHCHD10S59L expression imparts a toxic gain of function and that this dominant toxicity is mediated through two distinct axes: TDP-43 and PINK1. CHCHD10S59L expression increased insolubility and mitochondrial association of TDP-43 and also activated PINK1-mediated pathways. Pharmacologic treatments with peptide inhibitors of TDP-43 translocation to mitochondria or PINK1 kinase activity successfully mitigated degenerative phenotypes in HeLa cells. Small-molecule agonists of mitofusin (MFN), a downstream substrate of PINK1, rescued mutant CHCHD10-induced mitochondrial defects in Drosophila and HeLa cells. Moreover, the MFN agonists enhanced mitochondrial ATP production in a Drosophila ALS-FTD model expressing 901stf72 with expanded GGGGCC repeats. These findings suggest that CHCHD10S59L-induced ALS-FTD shares a disease-causing mechanism through mitochondrial translocation of TDP-43 with other subtypes of ALS-FTD and that modulating mitochondrial function by targeting PINK1-mediated pathways may provide a therapeutic strategy for CHCHD10-mediated disease.

Results

CHCHD2 and CHCHD10 share a common Drosophila melanogaster ortholog. To elucidate whether an ortholog of CHCHD10 exists in Drosophila, we used the Drosophila RNAi (RNA interference) Screening Center’s Integrative Ortholog Prediction Tool29. We found that Drosophila and humans (Homo sapiens) have three and two homologous genes for CHCHD10, respectively. Further phylogenetic analysis with a neighboring joining tree of these genes revealed that GS5010 is the Drosophila gene sharing the highest homology with both human CHCH2 and CHCHD10 (Supplementary Fig. 1a). Two additional Drosophila homologs, Dmel\CG31007 and Dmel\CG31008 appeared independently after their speciation. The substituted amino acids in human patients are conserved in Drosophila GS5010 (Fig. 1a). In addition, GS5010 is highly expressed in all Drosophila tissues, whereas Dmel\CG31007 and Dmel\CG31008 are expressed only in the testis and weakly in the imaginal discs. A comparison between H. sapiens and Mus musculus suggested that CHCHD10 and CHCHD2 were duplicated before their speciation and that they may be involved in common processes, but in a distinct manner30. According to its phylogenetic and expression profile, we deemed GS5010 as a common Drosophila orthology for both CHCHD10 and CHCHD2. Therefore, we hereafter refer to GS5010 as C2C10H (i.e., CHCHD2 and CHCHD10 homolog) and Dmel\CG31007 and Dmel\CG31008 as C2C10L1 (i.e., CHCHD2–CHCHD10-like 1) and C2C10L2 (i.e., CHCHD2 and CHHD10-like 2), respectively.

CHCHD10S59L causes dominant degeneration in fly eyes. To develop Drosophila models for mutant CHCHD10-induced human disease, we generated both Drosophila and codon-optimized human versions of transgenic animals carrying C2C10HW and S81L (CHCHD10WT and S59L in human), with
**Fig. 1** C2C10H^{S81L} is toxic in *Drosophila* eyes, neurons, and muscles. **a** Protein sequence alignment of human CHCHD10 and *Drosophila* C2C10H (CG5010). Disease-causing sites (asterisk) are conserved between human CHCHD10 and *Drosophila* C2C10H. **b** C2C10H^{S81L} (representative images from two independent experiments) and **c** human CHCHD10^{S59L} cause age-dependent mild rough eye phenotypes in 40-day-old flies (representative images from two independent experiments). Scale bar = 200 μm. **d** Representative images of neuromuscular junctions and crawling traces from the genotypes indicated (see below for statistical analysis). Scale bar = 20 μm. **e** Adult thoraxes dissected to expose longitudinal indirect flight muscles and stained with phalloidin-Alexa Fluor 594. Flies expressing C2C10H^{S81L} in muscles under control of MHC-GAL4 exhibit disrupted sarcomere structures. Scale bar = 0.5 mm (fly) and 10 μm (muscle). **f** Expression of C2C10H^{S81L} in motor neurons results in small synapses, with reduced bouton and branch numbers and defective locomotive activity assessed by the crawling behavior of third-instar larvae. Data are mean ± SD (one-way ANOVA and post hoc Dunnett test, two-sided, ***p = 5.3e − 10, 0.0002 and 3.8e − 14 for bouton number (n = 10–11), branch number (n = 12–14), and crawling distance (n = 18) from three independent experiments, respectively). **g** Expression of C2C10H^{S81L} in muscle tissues causes abnormal wing postures and locomotor defects assessed by flight ability. Data are mean ± SD (one-way ANOVA and post hoc Dunnett test, two-sided, ***p = 4.3e − 10, 5.5e − 05 for abnormal wing posture, flight assay, respectively; n = 4 with >40 flies).
or without a C-terminal FLAG tag by ΦC31 integrase-mediated site-specific integration into the attP2 landing site on the third chromosome. When C2C10H<sup>WT</sup> and CHCHD10<sup>WT</sup> were expressed in Drosophila eyes by the glass multimer reporter (GMR)-GAL4 driver, they did not cause any abnormal phenotypes at eclosion. Although C2C10H<sup>WT</sup> and CHCHD10<sup>WT</sup> expression did not cause any apparent defects, expression of C2C10H<sup>F81L</sup> and CHCHD10<sup>S59L</sup> caused mild but mutation-dependent degeneration with depigmentation as the flies aged, regardless of FLAG tagging (Fig. 1b, c and Supplementary Fig. 1d). The severity of rough eye phenotypes was determined by Flownyther scoring of the disorderliness of ommatidia<sup>1</sup>. There were statistically significant differences in eye phenotypes (Supplementary Fig. 1b, c, d) without substantial differences in the expression level of WT and mutant proteins (Supplementary Fig. 1e). When we generated fly lines carrying two copies of C2C10H<sup>F81L</sup>, at the attP2 locus with GMR-GAL4, the rough eye phenotype of C2C10H<sup>F81L</sup> was enhanced and obvious at eclosion due to high levels of expression by transversion<sup>32</sup> (Supplementary Fig. 1f, g). However, the expression of two copies of C2C10H<sup>WT</sup> did not induce any eye defects (Supplementary Fig. 1f). These findings indicate that overexpression of C2C10H<sup>F81L</sup> (CHCHD10<sup>S59L</sup>) generates mutant-dependent degeneration in Drosophila eyes.

C2C10H<sup>F81L</sup> recapitulates morphologic and functional defects in motor neurons and muscles. Patients carrying CHCHD10<sup>S59L</sup> experience motor neuron defects and myopathy. Thus, we examined whether C2C10H<sup>F81L</sup> and CHCHD10<sup>S59L</sup> expression cause degenerative phenotypes in Drosophila motor neurons and muscles. Due to the weaker cell toxicity of CHCHD10<sup>S59L</sup> compared to C2C10H<sup>F81L</sup> in the Drosophila system, C2C10H<sup>F81L</sup> was mainly used in most experiments. Expressing C2C10H<sup>WT</sup> or C2C10H<sup>F81L</sup> in motor neurons with OK371-GAL4 did not cause noticeable abnormalities in larvae or adult flies, including viability and fertility. However, homozygous animals for OK371-GAL4 and C2C10H<sup>F81L</sup> exhibited robust degenerative phenotypes, whereas homozygous C2C10H<sup>F81L</sup> flies with OK371-GAL4 did not show any abnormalities. Third-instar C2C10H<sup>F81L</sup> homozygous larvae showed striking locomotor dysfunction (Fig. 1d, f), with marked morphologic defects in their neuromuscular junctions (NMJs), including decreased synaptic bouton and branch numbers, and the absence of such NMJ defects in driver-only or C2C10H<sup>WT</sup>-expressing flies (Fig. 1d, f). Consistently, a locomotive defect was also observed with third-instar homozygous larvae expressing CHCHD10<sup>S59L</sup> in motor neurons (Supplementary Fig. 1h). Expression of C2C10H<sup>F81L</sup> but not C2C10H<sup>WT</sup> in indirect flight muscles with MHC-GAL4 led to mutant-dependent muscle degeneration, which was characterized by abnormal wing posture and loss of sarcomere architecture in aged flies (Fig. 1e, g). In addition, C2C10H<sup>F81L</sup> expression in muscles resulted in functional locomotor defects, as evident in flight assays (Fig. 1g). These findings reveal that the Drosophila model recapitulates the dominant cell toxicity of CHCHD10<sup>S59L</sup> in vivo.

CHCHD10<sup>S59L</sup> induces mitochondrial defects. CHCHD10 is primarily localized at cristae junctions in the mitochondrial intermembrane space<sup>1,33</sup>. To determine whether CHCHD10<sup>S59L</sup> induces mitochondrial defects, we transiently expressed CHCHD10<sup>WT</sup> or CHCHD10<sup>S59L</sup> tagged with C-terminal FLAG in HeLa cells. Immunocytochemistry using an anti-FLAG antibody revealed CHCHD10 localization in mitochondria but not in other cellular sites (Fig. 2a). Consistent with the previous reports<sup>1,19,34</sup>, expression of CHCHD10<sup>S59L</sup> caused notable mitochondrial fragmentation and functional respiratory defects, as measured by Seahorse XF Cell Mito Stress tests. In contrast, CHCHD10<sup>WT</sup> expressed at a similar level to CHCHD10<sup>S59L</sup> induced mitochondrial elongation and improved respiratory function over that of empty vector-transfected cells (Fig. 2a–c and Supplementary Fig. 2a). CHCHD10<sup>S59L</sup> displayed a punctate staining pattern in mitochondria, whereas CHCHD10<sup>WT</sup> staining was evenly distributed (Fig. 2a, b). It is unclear whether this punctate pattern is due to CHCHD10 aggregation or mitochondrial fragmentation because the staining patterns of the mitochondrial marker and CHCHD10<sup>S59L</sup> mostly overlapped (Fig. 2a, b).

To examine mitochondrial morphology and function in response to C2C10H<sup>F81L</sup> expression in Drosophila, we expressed two copies of C2C10H<sup>F81L</sup> in muscle tissues with the MHC-GAL4 driver. Visualization of mitochondria with a green fluorophore-conjugated streptavidin revealed that expression of C2C10H<sup>F81L</sup> in indirect flight muscles resulted in muscular degeneration, along with fragmented mitochondria. This finding was in contrast with our observations in the indirect flight muscles of MHC-GAL4 only or C2C10H<sup>WT</sup>-expressing flies (Fig. 2d). We measured ATP levels as an indicator of mitochondrial dysfunction in the flies’ thoraces, which contain primarily muscle tissues. We observed reduced ATP levels in the muscle tissues of flies expressing C2C10H<sup>F81L</sup>, as compared with those of MHC-GAL4/+ control flies (Fig. 2e). Consistent with the effect of CHCHD10<sup>WT</sup> overexpression in HeLa cells, C2C10H<sup>F81L</sup> expression also increased ATP levels in Drosophila muscle tissues (Fig. 2e). Consistently, expression of CHCHD10<sup>S59L</sup> protein in Drosophila muscles caused similar defects (Supplementary Fig. 2d). Therefore, C2C10H<sup>F81L</sup> and CHCHD10<sup>S59L</sup> induced consistent mitochondrial fragmentation and functional respiratory defects in Drosophila and mammalian cells.

CHCHD10<sup>S59L</sup> is not a hypermorphic gain-of-function mutant. The S59L substitution in CHCHD10 is dominantly inherited<sup>1</sup>. The dominant cell toxicity imparted by CHCHD10<sup>S59L</sup> overexpression suggests two possible modes of action: dominant negative or dominant gain of function. There are also two possible modes for gain of function: gain of a WT function (hypermorph) or gain of an abnormal function (neomorph)<sup>85</sup>. First, we tested if CHCHD10<sup>S59L</sup> is a hypermorph. If CHCHD10<sup>S59L</sup> enhances its own functions to generate cell toxicity, CHCHD10<sup>WT</sup> co-expression with CHCHD10<sup>S59L</sup> should enhance the degenerative phenotype of CHCHD10<sup>S59L</sup>. For this genetic interaction study using C2C10H<sup>F81L</sup>, we generated a fly model carrying two copies of C2C10H<sup>F81L</sup> in the second and the third chromosomes. When the two C2C10H<sup>F81L</sup> copies were expressed via GMR-GAL4, it induced relatively mild rough eye phenotypes at eclosion (Fig. 3a) compared with that of third chromosome homozygotes (Supplementary Fig. 1f). We tested several different control Drosophila lines with this model fly and did not observe the significant modification of phenotypes by the control lines (Supplementary Fig. 3a). Significantly, C2C10H<sup>WT</sup> co-expression did not enhance but suppressed the C2C10H<sup>F81L</sup>-induced rough eye phenotype (Fig. 3a), in addition to the morphologic and functional mitochondrial defects in both Drosophila (Fig. 3b, c) and HeLa cells (Fig. 3d, e and Supplementary Fig. 3c). C2C10H<sup>F81L</sup>-induced rough eye phenotypes were also not enhanced but rescued by human CHCHD10<sup>WT</sup> co-expression (Supplementary Fig. 3e). This suggests that CHCHD10<sup>S59L</sup> is not a hypermorph enhancing its own WT activity. To exclude the possibility of unknown positional effects such as transvection, we generated another transgenic fly line with C2C10H<sup>WT</sup> or C2C10H<sup>F81L</sup> in an unrelated landing site, VK27. C2C10H<sup>WT</sup> VK27 also rescued the C2C10H<sup>F81L</sup> phenotypes, whereas C2C10H<sup>F81L</sup> VK27 enhanced the rough eye phenotype.
Consistent with our observations in Drosophila eyes, co-expression of C2C10WT mitigated the C2C10S81L-induced mitochondrial fragmentation and ATP production defects in indirect flight muscles (Fig. 3b, c). An additional copy of C2C10S81L exacerbated the defects in both the eyes and muscles (Fig. 3a–c).

In HeLa cells, co-expression of CHCHD10WT with CHCHD10S59L clearly mitigated morphologic (Fig. 3d, e) and functional defects (Supplementary Fig. 3c). However, the punctate staining pattern of CHCHD10S59L was not altered by CHCHD10WT co-expression (Fig. 3d, e), indicating that CHCHD10WT improves mitochondrial integrity by a mechanism other than restoring mutant protein aggregation. Immunostaining of indirect flight muscles revealed that C2C10H8S11 and CHCHD10S59L proteins also formed aggregate-like structures in vivo (Supplementary Fig. 2b, c). Together, these results suggest that C2C10H8S11 and CHCHD10S59L clearly form aggregates and do not act as a hypermorph (gain of WT function).

**CHCHD10S59L is a dominant gain-of-function mutant.** The mislocalized punctate pattern of CHCHD10S59L (C2C10H8S11) suggests that CHCHD10S59L (C2C10H8S11) may be a dominant gain-of-toxic (neomorphic) mutant acquiring abnormal functions. To distinguish a dominant gain-of-toxic mutant from a dominant-negative mutant suppressing the activity of its WT form, we tested whether the toxicity of CHCHD10S59L and C2C10H8S11 is dependent on the existence of CHCHD10WT and CHCHD10S59L. The mislocalized punctate pattern of CHCHD10S59L (C2C10H8S11) suggests that CHCHD10S59L (C2C10H8S11) may be a dominant gain-of-toxic (neomorphic) mutant acquiring abnormal functions. To distinguish a dominant gain-of-toxic mutant from a dominant-negative mutant suppressing the activity of its WT form, we tested whether the toxicity of CHCHD10S59L and C2C10H8S11 is dependent on the existence of CHCHD10WT and CHCHD10S59L.
C2C10HWT, respectively. If they are true dominant negative, the phenotype of C2C10H deletion mutant (C2C10Hnull) animals and CHCHD10 knockout (CHCHD10KO) cells will not be enhanced by the expression of C2C10HS81L and CHCHD10S59L. Because C2C10Hnull flies do not exhibit an abnormal eye phenotype and are generally healthy, we expected that the C2C10HS81L-induced rough eye phenotype would not be present in the C2C10Hnull background if it is a dominant-negative mutant. However, the rough eye phenotype was robust in the C2C10Hnull background (Fig. 3f). Although we tested if two paralogs of C2C10H (Dmel\CG31007 and Dmel\CG31008) can modify the C2C10HS81L-induced phenotypes by RNAi-mediated knockdown
(Supplementary Table 1), we did not observe any significant interaction as they are not well expressed in Drosophila tissues except testis. Together, these results suggest that C2C10HWT is not a dominant-negative mutant requiring C2C1OH to generate its cell toxicity.

To further validate this result in a mammalian system, we generated CHCHD10KO HeLa cells via the CRISPR/Cas9 system (Supplementary Fig. 3f). Although the CHCHD10 protein was not detected with an anti-CHCHD10 antibody in the CHCHD10KO lines, mitochondrial morphology and respiratory functions were not affected (Fig. 3g, i and Supplementary Fig. 3g). Consistent with results from our Drosophila model, the expression of CHCHD10S59L-induced CDCCD10WT-independent mitochondrial toxicity in CHCHD10KO HeLa cells (Fig. 3g, i and Supplementary Fig. 3h). To further investigate the possibility that CHCHD10S59L might suppress a close paralog CHCHD2 activity to generate the mitochondrial toxicity in a dominant-negative manner, we generated CHCHD2 and CHCHD10 double-knockout (CHCHD2/10DKO) cells (Supplementary Fig. 3f). Consistent with a previous report34, mitochondrial morphology and respiratory functions were not affected in CHCHD2/10DKO cells (Fig. 3h, i and Supplementary Fig. 3g). The expression of CHCHD10S59L in CHCHD2/10DKO cells also generated significant morphological and functional defects in mitochondria (Fig. 3h-j and Supplementary Fig. 3h). All these data support that CHCHD10S59L mutation is a dominant gain-of-function (i.e., toxic) mutation causing aggregation of the mutated protein, not a dominant-negative mutant suppressing the activity of its WT counterpart.

CHCHD10S59L proteins form insoluble aggregates. The punctate staining pattern of CHCHD10S59L was not affected when CHCHD10WT rescued CHCHD10S59L-induced mitochondrial fragmentation. The S59L substitution is located in the hydrophobic domain of an intrinsically disordered region in CHCHD10 (Supplementary Fig. 4a). Because the hydrophobic domain and intrinsically disordered region play a role in protein folding and aggregation, we examined whether CHCHD10S59L proteins accumulate in mitochondria as relatively insoluble aggregates. We detected insoluble CHCHD10S59L proteins (i.e., RIPA-insoluble and urea-soluble) by immunoblotting (Fig. 4a). Co-expression of CHCHD10WT with CHCHD10S59L did not suppress the accumulation of insoluble CHCHD10S59L (Fig. 4a), which was consistent with the presence of punctate structures of CHCHD10S59L in elongated mitochondria by co-expressing CHCHD10WT (Fig. 3d, e). Therefore, CHCHD10S59L clearly forms insoluble aggregates in mitochondria. Again, CHCHD10WT co-expression did not affect the insolubility and aggregate formation. However, it is still unclear whether the presence of insoluble CHCHD10S59L is more relevant to toxicity than that of other intermediate species, such as oligomers.

CHCHD10S59L induces TDP-43 insolvency and mitochondrial translocation. Mutations in TARDBP cause ALS and FTD, and cytoplasmic TDP-43 aggregates are a hallmark in most patients with ALS and/or FTD37,38. In addition, mitochondrial translocation of TDP-43 and toxicity is reported in cell cultures and patient tissues25. Therefore, we hypothesized that CHCHD10 affects TDP-43 aggregation or mitochondrial translocation. We examined insoluble TDP-43 levels after CHCHD10WT and CHCHD10S59L transfection. Strikingly, CHCHD10S59L expression increased insoluble TDP-43, whereas CHCHD10WT expression decreased insoluble TDP-43 (Fig. 4b). Co-expression of CHCHD10WT with CHCHD10S59L suppressed CHCHD10S59L-induced insoluble TDP-43 (Fig. 4b).

We next determined whether CHCHD10WT and CHCHD10S59L expression affects mitochondrial translocation of TDP-43. Despite similar expression levels in total lysates, exogenously expressed TARDBPWT and three pathogenic mutants (G298S, A315T, and A382T) showed an elevated amount in the mitochondrial fraction of CHCHD10S59L-expressing cells over that of empty vector-transfected cells. We observed decreased mitochondrial distribution of TDP-43 WT and its mutants in CHCHD10WT-expressing cells (Fig. 4c, d). Furthermore, co-expression of CHCHD10WT with CHCHD10S59L reduced the amount of TDP-43 and its mutants in the mitochondrial fraction, which was increased by CHCHD10S59L expression (Fig. 4e, f). Immunofluorescence staining confirmed the increased mitochondrial localization of TDP-43 in CHCHD10S59L-expressing HeLa cells with transiently transfected...
TDP-43 (Supplementary Fig. 4b). In addition, increased cytoplasmic and mitochondrial localization of endogenous TDP-43 was observed in SH-SY5Y neuroblastoma cells expressing CHCHD10S59L (Supplementary Fig. 4c, d). An in vivo experiment with a humanized Drosophila model replacing the entire TBPH coding region with human TARDBP39 revealed significantly increased TDP-43 association with mitochondria in C2Cl0H59L-expressing muscle tissues, as compared to C2Cl0HWT-expressing muscles (Supplementary Fig. 4e). These observations suggest that the prevention of TDP-43 mitochondrial translocation, as demonstrated by Wang et al.25, may be a potential therapeutic strategy for CHCHD10-induced ALS-FTD. To test whether
inhibition of TDP-43 mitochondrial translocation recovers CHCHD10S59L-induced mitochondrial morphologic and functional defects, we treated CHCHD10S59L-transfected cells with the PM1 peptide inhibitor of TDP-43 mitochondrial translocation. Notably, the morphologic and functional defects caused by CHCHD10S59L were ameliorated by PM1 (Fig. 4g, h and Supplementary Fig. 4f), suggesting that increased mitochondrial translocation are critical to CHCHD10-induced cell toxicity. Although it has been demonstrated that TDP-43 can translocate into mitochondria in diseases or stressed conditions, it is not known how TDP-43 can be abnormally retained in mitochondria upon CHCHD10 mutation. We hypothesized that CHCHD10S59L increases the binding capacity of CHCHD10 to TDP-43 in mitochondria because CHCHD10 lacks catalytic activity, and physical interactions between CHCHD10 and TDP-43 are reported. To test this hypothesis, HEK293T cells were transfected with CHCHD10WT–FLAG or CHCHD10S59L–FLAG with/without TDP-43–tomato–HA, followed by immunoprecipitation with anti-FLAG affinity beads. Significantly, CHCHD10S59L showed an increased binding capacity to TDP-43 (Fig. 4i and Supplementary Fig. 4g). This was reaffirmed by using another combination of purification tags: CHCHD10–HA and TDP-43–FLAG (Supplementary Fig. 4h).

PINK1/parkin mediates dominant degeneration in the C2C10H581L Drosophila model. Because C2C10H581L and CHCHD10S59L expression caused consistent mitochondrial toxicity in Drosophila and human cells, respectively, we have further dissected genetic pathways of C2C10H581L-mediated cell toxicity using the Drosophila model. Because of notable mitochondrial fragmentation and functional respiratory defects, we hypothesized that the genes involved in mitochondrial dynamics or mitochondrial quality control are effectors of CHCHD10S59L-driven mitochondrial pathogenesis. We performed genetic interaction studies using the Drosophila C2C10H581L model with various RNAi, classical deficiency, and duplication lines (Supplementary Table 1). The most potent dominant suppressor was PINK1. Downregulation of PINK1 by RNAi recovered the rough eye phenotype produced by two copies of C2C10H581L, whereas PINK1 overexpression enhanced the rough eye phenotype (Fig. 5a). Moreover, RNAi-mediated depletion of parkin, a downstream partner of PINK1 for the mitochondrial quality control, also marginally rescued the rough eye phenotype (Fig. 5a).

We also tested several other genes that are parallel or downstream of the PINK1/parkin pathway, such as mul1, aril, march5, Drp1, marf, and TER94 (Supplementary Table 1). Only overexpression of marf, a Drosophila ortholog of MFN (i.e., mitofusin), showed mild suppressive effects with consistent RNAi-mediated enhancement (Fig. 5a). This indicates that Marf may be a downstream effector of the PINK1/parkin pathway during CHCHD10S59L-mediated pathogenesis. However, this observation does not exclude the other genes tested from playing a role in C2C10H581L-mediated pathogenesis. Consistent with our findings in the eye model, RNAi-mediated depletion of PINK1 also recovered C2C10H581L-dependent indirect flight muscle degeneration (Fig. 5b). PINK1 knockdown rescued sarcomere structure, extended mitochondrial length, and increased ATP production (Fig. 5b, c), improving C2C10H581L-induced defects, including abnormal wing posture and flight ability (Supplementary Fig. 5a). Human CHCHD10S59L-induced rough eye phenotypes were also rescued by PINK1 knockdown. (Supplementary Fig. 5b). These results suggest that C2C10H581L (and human CHCHD10S59L) activates the PINK1/parkin pathway to generate dominant cell toxicity in the Drosophila system.
In our *Drosophila* model, both Drp1 knockdown and overexpression exhibited detrimental effects (Supplemental Table 1). However, RNAi-mediated depletion of DRP1 in HeLa cells successfully reversed *CHCHD10*<sup>S59L</sup>-induced mitochondrial fragmentation and exerted beneficial effects on respiratory function (Supplementary Fig. 5i, j). Consistently, *CHCHD10*<sup>S59L</sup>-mediated respiratory defects were slightly enhanced by DRP1-YFP co-expression, although it was not statistically significant. (Supplementary Fig. 5h). Indeed, *CHCHD10*<sup>S59L</sup> aggregates were nearly absent with DRP1 knockdown (Supplementary Fig. 5g, i), in contrast with persistent aggregates with PINK1 knockdown (Fig. 5d, e). We speculated that pre-elongated mitochondria with DRP1 knockdown reduces the local concentration of *CHCHD10*<sup>S59L</sup> proteins and thus prevents aggregate formation. Although it is apparent that modulation of mitochondrial dynamics may be beneficial in reducing...
Fig. 5 CHCHD10S59L-induced degeneration is rescued by Pink1 downregulation. a RNAi-mediated knockdown effects of PINK1, Parkin, and their downstream target, Marf (Drosophila mitofusin) on CZC10S59L-induced eye phenotypes. Boxes indicate median and 25th and 75th percentiles (one-way ANOVA and post hoc Dunnett test, two-sided, or Fisher’s least significant difference, \( ^* p = 1.3e^{-06} \), 0.0004 and \( p = 0.0486 \) in KD, \( p = 0.04797 \), and \( p = 0.00015 \) in OE for PINK1, parkin and Marf, respectively; n = 4–11 flies from a single experiment; results were verified with multiple fly lines; see Supplementary Table 1). Scale bar = 200 μm. b RNAi-mediated knockdown of PINK1 in muscles rescued CZC10S59L-induced muscle degeneration and mitochondrial defects. Representative images from three independent experiments. Scale bar = 20 μm. c ATP levels in thoraxes from 10-day-old flies were measured. Data are shown as mean ± SD (one-way ANOVA and post hoc Dunnett test, \( ^* p < 2e^{-16} \); n = 20 cells from three independent experiments). d Mitochondrial respiration was measured by Seahorse XF Cell Mito Stress tests 24 h after CHCHD10S59L transfection into siRNA-treated cells. Graphs of a single representative experiment are shown (mean ± SD). Actual statistical analyses were performed with 8 independent experiments (one-way ANOVA and post hoc Dunnett test, two-sided, comparison with EV; \( ^* * p < 0.0034 \), 0.00031 and \( ^* * * p = 0.0055 \) for basal, ATP and maximal level, respectively; detailed information on statistical analyses is available in Supplementary Fig. 9g). e RT-qPCR analysis of PINK1 in siRNA-transfected fibroblasts. Results are shown as fold change of PINK1 mRNA expression in siPINK1 transfected fibroblasts relative to siControl transfected fibroblasts. Data were normalized to β-2-macroglobulin (B2M) or 28S. Results shown are mean ± SEM from two independent experiments. f Pink1 downregulation by siRNA treatment reversed mitochondrial network fragmentation in CHCHD10S59L+/− patient fibroblasts. Patients (P1, P2) or wild-type (WT) fibroblasts were transfected with control siRNA (siCont) or PINK1 siRNA (siPINK1). P1 and P2 correspond to patient V-101 and to patient IV-311, respectively. Representative images of the mitochondrial network with MitoTracker staining. Scale bar = 20 μm. Mitochondrial network length was quantified from two independent experiments with 48–116 randomly selected individual cells. Differences between siCont and siPINK1 were analyzed by two-sided Mann-Whitney test (\( ^* * p = 0.0024 \) and 0.0013 for patients 1 and 2, respectively).

CHCHD10S59L–induced cell toxicity, modulating PINK1, parkin, or MFN is less detrimental than directly modulating DRP1. Finally, we examined whether RNAi-mediated downregulation of PINK1 affects the fragmented mitochondrial network observed in fibroblasts derived from patients carrying a CHCHD10S59L allele.11,12. Transfection with PINK1 small interfering RNA (siRNA) successfully reduced the amount of PINK1 transcripts in both control and patient fibroblasts (Fig. 5g). MitoTracker staining and analysis for control and PINK1 siRNA-transfected cells showed that the loss of PINK1 rescued the fragmented mitochondrial network found in patient-derived fibroblasts (Fig. 5h).

Parkin-mediated mitophagy induces cell toxicity. Upon mitochondrial stress or damage, PINK1 accumulates in mitochondria42 and recruits parkin by phosphorylating ubiquitin and other substrates, including parkin, resulting in MFN1/2 degradation and mitophagy to remove damaged mitochondria43–46. The suppressive effects of PINK1 and PRKN knockdown suggest that CHCHD10S59L induces PINK1 accumulation in mitochondria, activating the PINK1/parkin pathway. Indeed, PINK1-YFP accumulated in the mitochondria of CHCHD10S59L-expressing HeLa cells, in contrast with that of CHCHD10WT overexpression (Fig. 6a, c). Because PRKN expression is deficient in HeLa cells, stable cell lines expressing YFP-Parkin (HeLaYFP-Parkin) have been established and widely used to study parkin-mediated mitophagy.42 When CHCHD10S59L was transiently expressed in HeLaYFP-Parkin cells, YFP-Parkin was also recruited to mitochondria (Fig. 6b, d), clearly demonstrating that CHCHD10S59L induces PINK1 stabilization, accumulation on mitochondria, and subsequent parkin recruitment. This suggests that PINK1/parkin-mediated mitophagy is toxic in this system. Indeed, enhancing autophagy by Atg1 expression in the CZC10H881L eye model was synergistically lethal (Supplementary Table 1), although beneficial effects of Atg1 overexpression have been reported in other Drosophila disease models.47,48 However, strong mitochondrial abnormalities in PRKN-deficient HeLa cells also indicate the presence of parkin-independent toxic mechanisms. To explain CHCHD10S59L–induced cell toxicity in both PRKN-deficient HeLa cells and HeLaYFP-Parkin, we hypothesized that PINK1-mediated, parkin-independent mitophagy49 is highly activated in PRKN-deficient HeLa cells by CHCHD10S59L and that deregulated mitophagy is amplified by Parkin in HeLaYFP-Parkin.

To test this hypothesis, we first assessed LC3 conversion and accumulation in mitochondria to examine whether autophagosome formation is activated by CHCHD10S59L expression in HeLa cells. In both HeLa and HeLaYFP-Parkin cells, CHCHD10S59L expression increased LC3 accumulation (Fig. 6e, f) and LC3 conversion (Fig. 6g). However, only small portions of LC3 accumulation colocalized with mitochondria in PRKN-deficient HeLa cells, as compared with strong mitochondrial colocalization of LC3 accumulation in HeLaYFP-Parkin cells (Fig. 6e, h). Co-staining of lysosomes and mitochondria also revealed limited lysosomal marker staining in the mitochondria of PRKN-deficient Hela cells (Supplementary Fig. 6a). To confirm this observation, we determined the mitochondrial LC3 levels after fractionating mitochondria. Although LC3-II amount increased in mitochondrial fractions of CHCHD10S59L–transfected HeLa and HeLaYFP-Parkin, significantly more LC3-II were detected in HeLaYFP-Parkin as expected (Supplementary Fig. 6b). A mitochondrial fraction from CHCHD10S59L–transfected SH-SY5Y expressing parkin endogenously showed clear LC3-II accumulation (Supplementary Fig. 6c). To determine whether the increased LC3 levels from CHCHD10S59L–transfected cells correspond with mitophagic turnover, we examined mitolysosomes using the mito-QC system in HeLa, HeLaYFP-Parkin, and SH-SY5Y cells. Consistent with LC3 conversion and accumulation, mito-QC-positive mitolysosomes (GFP-negative/mCherry-positive) were increased in CHCHD10S59L–expressing cells, especially in HeLaYFP-Parkin and SH-SY5Y (Fig. 6i, j and Supplementary Fig. 6d, e). Therefore, it is apparent that mitophagy induction is a major phenomenon when CHCHD10S59L is expressed. However, it is still unclear whether PINK1-mediated, parkin-independent mitophagy mildly induced by CHCHD10S59L contributes to the cell toxicity in PRKN-deficient HeLa cells. Thus, to test whether the induction of PINK1-mediated, parkin-independent mitophagy by CHCHD10S59L is critical for generating cell toxicity in PRKN-deficient HeLa cells, we reduced the expression of two mitophagy receptors involved in PINK1-mediated, parkin-independent mitophagy, NDP52 and optineurin (OPTN)49 by RNAi. Knockdown of these two receptors had no effect on CHCHD10S59L–mediated cell toxicity in PRKN-deficient HeLa cells, but increased respiratory activity significantly in HeLaYFP-
Parkin cells (Fig. 6k, l and Supplementary Fig. 6f–h). Therefore, the PINK1/parkin-mediated mitophagy pathway is one of the major toxicogenic pathways when Parkin exists. However, without Parkin, the mitophagic pathway is not essential to generate the substantial cell toxicity observed in PRKN-deficient HeLa cells, indicating that PINK1 downstream factors other than Parkin can also mediate the cell toxicity independent of Parkin.

Modulating PINK1 downstream pathways mitigates CHCHD10S59-induced cell toxicity. To further define the
Parkin cells were transfected with siRNAs targeting mCherry-LC3 and Tom20 merged region. Scale bar and HeLaYFP-Parkin cells. Co-localization was measured by Pearson Dunnett test, two-sided, ** respectively. Representative images of transfected cells immunostained with antibodies against FLAG and TOM20. Graphs show the number of cells showing GFP- or mCherry-LC3 accumulation. Data are shown as mean ± SD (one-way ANOVA and post hoc Dunnett test, two-sided, *** comparison with empty vector [EV]; n = 3 independent experiments).

We treated HeLa cells with two putative peptide inhibitors of CHCHD10S59L-induced mitochondrial fragmentation was compared to wild-type (WT) and TDP-43A382T were unaffected in HeLa cells (Fig. 8a, b). In addition, insoluble CHCHD10S59L and TDP-43 levels were not changed by PINK1 knockdown (Fig. 8c, d), suggesting that PINK1 accumulation in response to CHCHD10S59L expression is parallel or downstream (or both) of the TDP-43 pathway. CHCHD10WT co-expression with CHCHD10S59L reduced PINK1 accumulation (Fig. 8e). Intriguingly, CHCHD10WT expression also reduced PINK1 and parkin accumulation caused by mild CCCP treatment in HeLa and HeLaYFP-Parkin cells, respectively (Fig. 8f and Supplementary Fig. 8a, b). Therefore, CHCHD10WT protected mitochondria not only through TDP-43 but also by preventing PINK1 accumulation. CHCHD10WT exerted a protective effect through both TDP-43 and PINK1 without modulating CHCHD10S59L insolubility (Fig. 9), suggesting that augmenting CHCHD10 expression or activity is also a promising therapeutic strategy, in addition to specifically blocking TDP-43 mitochondrial translocation and/or PINK1 activity.

Discussion
Since the initial identification of the S59L substitution in CHCHD10, additional variants have been identified and suggested as pathogenic mediators of ALS-FTD, SMAJ, and mitochondrial myopathy. Despite efforts to elucidate their pathogenic mechanisms, many controversial findings suggest that mutations in CHCHD10 do not share a common disease-causing mechanism.11,12,19–22 We found that only CHCHD10S59L induced the dominant dominant cell toxicity in both Drosophila and HeLa cells.35

Consistent with recent studies demonstrating a dominant mechanism of CHCHD10S59L in CHCHD10KO mice and CHCHD10S59L knockin mice,56,57 our own findings do not support simple loss-of-function or haploinsufficiency mechanisms. Woo et al. previously proposed a dominant-negative mechanism of CHCHD10S59L in Caenorhabditis elegans and mammalian systems. Interestingly, their observations are similar to our findings. However, our data support a dominant gain-of-toxic function mechanism for CHCHD10S59L, whereas Woo et al. proposed...
a dominant-negative mechanism, although they also observed results that can support a dominant gain-of-toxic function mechanism. Although these two mechanisms are not necessarily mutually exclusive, several aspects of our findings primarily support a dominant gain-of-toxic function mechanism. First, the Drosophila eye phenotypes driven by C2C10H59L did not differ between the C2C10Hnull or C2C10HWWT background, indicating that the mutations exert a dominant gain-of-toxic function rather than suppressing WT activity as a dominant negative. Second, CHCHD10S59L is toxic regardless of the presence of CHCHD10WT (and CHCHD2WT). Third, we confirmed the genetic modifiers modulating the dominant gain-of-toxic function in Drosophila in mammalian cells, as well. Fourth, parkin accumulation did not occur in CHCHD10KO HeLaSMC-Parkin cells, but it did occur with CHCHD10S59L expression35, indicating that the cell toxicity caused by reduced CHCHD10 differs from that of CHCHD10S59L.
Fifth, CHCHD10WT did not accumulate in CHCHD10S59L aggregate-like structures. Sixth, CHCHD10WT overexpression did not affect CHCHD10S59L aggregate formation and insolubility. Therefore, both CHCHD10WT and CHCHD10S59L are involved in the same pathway, independently in reverse directions, because CHCHD10 has a protective role in mitochondria that is also protective for CHCHD10S59L-driven dominant cell toxicity, which occurred independently of disrupting WT activity. However, we cannot completely rule out the contribution of loss-of-function- or dominant-negative-like effects. We anticipate that some effect of reduced WT activity occurs in disease pathogenesis. Our data suggest that the dominant cell toxicity of CHCHD10S59L can be mitigated by co-expressing similar levels of CHCHD10WT. Therefore, it is possible that a heterozygous CHCHD10S59L mutation does not induce severe degeneration, as long as WT activity blocks mutant cell toxicity. However, an age-dependent reduction of WT function or a change in the ratio of WT to mutant expression may trigger disease symptoms later. Taken together, multiple or possibly mixed mechanisms may exist, and CHCHD10S59L may cause a strong dominant phenotype that can be successfully modeled in both Drosophila and HeLa cells.

Our efforts to define the downstream pathways of dominant CHCHD10S59L-mediated cell toxicity yielded two axes and multiple molecular targets that can be therapeutically modulated (Fig. 9). The mitochondrial translocation of TDP-43 is a toxicity-generating mechanism in CHCHD10S59L-expressing cells. Wang et al. demonstrated that excess TDP-43 mitochondrial translocation induces mitochondrial dysfunction, and blocking this translocation abolishes mitochondrial toxicity. CHCHD10S59L induced TDP-43 mitochondrial translocation and inhibiting this translocation mitigated CHCHD10S59L-induced mitochondrial abnormalities. The association between TDP-43 and CHCHD10 was previously proposed, and Wang et al. raised a question for the importance of the mitochondrial TDP-43. In support of this, we showed that CHCHD10S59L bound to TDP-43 more greatly than did CHCHD10WT and that inhibition of TDP-43 mitochondrial translocation mitigated the CHCHD10S59L-induced phenotype. In addition, the effects of CHCHD10WT on CHCHD10S59L-induced toxicity and translocation generally support that TDP-43 is a key effector generating mitochondrial toxicity in ALS-FTD.

We identified PINK1 and PRKN as strong genetic modifiers of C2C10H5811-mediated cell toxicity. PINK1/parkin-mediated expression are generally regarded as protective for cells by removing damaged mitochondria. However, CHCHD10S59L expression induced PINK1 stabilization in mitochondria, and genetic/pharmacologic inhibition of PINK1 clearly mitigated CHCHD10S59L-mediated toxicity. Previous reports showed that reducing PINK1 or parkin-mediated pathways are beneficial in in vivo disease models of SOD1, FUS, and TARDBP mutations. We demonstrated that MFN2 agonists enhanced PINK1 activation in flies expressing the GGGGCC repeats of C9ORF72.

Two downstream phosphorylation substrates of PINK1, MFN, and mitofusin mediated CHCHD10S59L-induced cell toxicity. Although fusion activity in CHCHD10S59L-expressing cells is not altered, our data indicate that PINK1 accumulation and subsequent inactivation of MFN by phosphorylation is responsible for fragmented mitochondria and the respiratory defects caused by CHCHD10S59L. An MFN2 agonist developed for CMT2 was also effective for CHCHD10S59L-mediated cell toxicity in this context and may also be effective for the CHCHD10G66V mutation causing SMAJ or CMT2. Overexpression of a PINK1 phosphorylation-null MitoMorph mutant also rescued C2C10H5811-mediated cell toxicity, suggesting that deformation of the MICOS complex was not based on a direct interaction, but through phosphorylation by PINK1. Therefore, the degree of PINK1 stabilization may correspond with the phenotypic severity of mutant CHCHD10.

While this manuscript was under review for publication, two independent studies reported CHCHD10S59L-mediated OMA1 peptidase activation, subsequent degradation of OPA1 resulting in mitochondrial fragmentation, and a protective effect of CHCHD10WT against TDP-43 mitochondrial accumulation. Because OMA1 and PINK1 can be activated in the same experimental conditions, elucidating the association between the OMA1–OPA1 pathway and the PINK1-mediated pathway in CHCHD10S59L-induced pathogenesis will be worthwhile. Although we did not observe any meaningful protective effects of CHCHD10WT against toxic TDP-43 in Drosophila, this may be due to the strong overexpression of TDP-43 in Drosophila. Therefore, investigating the protective role of CHCHD10 in ALS-FTD and other degenerative diseases with mitochondrial defects is important. In contrast with our findings, two studies reported that their findings were also observed in multiple CHCHD2 and CHCHD10 double-knockout models or by CHCHD10 knockdown in cell culture. These findings combined with our data suggest that CHCHD10S59L-induced gain-of-toxic function, partial loss of CHCHD10WT, and dominant-negative-like inhibition of CHCHD2 may coexist during disease pathogenesis or individually contribute to specific aspects of disease pathogenesis.
Fig. 8 Dominant toxicity of CHCHD10S59L is mediated independently by TDP-43 and PINK1 signaling. a HeLa cells transfected with PINK1 siRNA were co-transfected with empty vector (EV) or FLAG-tagged CHCHD10S59L and FLAG-tagged TARDBPWT. Fractionated mitochondria were analyzed with anti-TDP-43 (arrowhead indicates transfected TDP-43) or anti-HSP60 and anti-Cox2 (loading controls) antibodies. Data are shown as mean ± SD (two-sided t test, NS = not significant; n = 4 independent experiments). b HeLa cells transfected with PINK1 siRNA were co-transfected with empty vector (EV) or FLAG-tagged CHCHD10S59L and TARDBPΔ382T. Fractionated mitochondria were analyzed with anti-TDP-43 (arrowhead indicates transfected TDP-43) or anti-HSP60 and anti-Cox2 (loading controls) antibodies. c HeLa cells transfected with PINK1 siRNA were transfected with FLAG-tagged CHCHD10S59L. RIPA-soluble and insoluble fractions were analyzed with anti-FLAG and anti-actin (loading control) antibodies. Data are shown as mean ± SD (two-sided t test, NS = not significant; n = 3 independent experiments). d HeLa cells transfected with PINK1 siRNA were co-transfected with FLAG-tagged CHCHD10S59L and TARDBP. RIPA-soluble and insoluble fractions were analyzed with anti-TDP-43 and anti-actin (loading control) antibodies. Data are shown as mean ± SD (two-sided t test, NS = not significant; n = 4 independent experiments). e HeLa cells transfected with PINK1-YFP, FLAG-tagged CHCHD10S59L, and HA-tagged CHCHD10WT or EV were visualized with anti-FLAG (green) and anti-V5 (red) antibodies to visualize CHCHD10 and PINK1, respectively. Arrow indicates PINK1 accumulated in a non-transfected cell neighboring a CHCHD10-transfected cell (white dashed line). The percentage of PINK1-positive cells from the empty vector (EV) or CHCHD10WT-transfected cells were calculated after 5 or 10 μM CCCP treatment for 6 h. Data are shown as mean ± SD (two-sided t test, **p = 0.009055 for 5 μM and 0.009203 for 10 μM CCCP treatment; n = 3 independent experiments, >200 cells for each group). Scale bar = 20 μm.
TDP-43 becomes more insoluble in the cytoplasm, probably due to mitochondria-induced stresses\textsuperscript{56,57} and accumulates in mitochondria via increased binding with CHCHD10\textsubscript{59L}. The toxicity generated by mitochondrial TDP-43 can be mitigated by inhibitors for TDP-43 mitochondrial translocation. PINK1 (Roche Applied Science) on a Light Cycler LC480. Results were normalized to performed in triplicate. QRT-PCR was carried out using SYBR Green Master Mix reverse transcribed using transcription... expression may mitigate such mitochondrial defects.

Augmenting CHCHD10\textsuperscript{WT} activity may be a promising therapeutic strategy, regardless of specific CHCHD10 mutations. CHCHD10\textsuperscript{WT} expression is increased in response to various stresses\textsuperscript{43}. We and others observed that CHCHD10\textsuperscript{WT} expression not only rescued mutant phenotypes but also increased mitochondrial length and respiratory activity when it was expressed alone in both Drosophila and HeLa cells. Although the mechanism by which CHCHD10 enhances mitochondrial function and reduced PINK1-mediated cell toxicity is not clear, our findings support that pharmacologic or epigenetic augmentation of CHCHD10 expression may mitigate such mitochondrial defects.

**Methods**

**Chemicals and peptides.** CCCP was purchased from Sigma-Aldrich. The MFN agonists B1 and B/A-\textsubscript{L} were described previously\textsuperscript{51}. ScPM and PM1 peptides for TDP-43 were kindly provided by Xinglong Wang. Pink1_490 and Ub_60 peptides were synthesized and inserted in the pcDNA3 vector containing a FLAG tag by Genescripts. All complementary DNAs (cDNAs) for human CHCHD10\textsubscript{WT} and variants were synthesized and inserted in the pcDNA3 vector containing a FLAG, HA, or Myc tag by Genescripts. mTagRFP-T-Mito-7 (\#58023)\textsuperscript{34}, TDP43tdTomATO-HA (\#28025)\textsuperscript{35}, EGF-C-LC3 (\#21073)\textsuperscript{36}, mCherry-LC3B (\#0827)\textsuperscript{37}, pEYFP-C1-DRP1 (\#45160)\textsuperscript{38}, pEYFP-N1-PINK1 (\#101574)\textsuperscript{52}, and LAM1-mGFP (\#3831)\textsuperscript{53} plasmids were obtained from Addgene. FLAG-MFN2\textsubscript{WT}, FLAG-MFN2\textsubscript{S574A}, and FLAG-MFN2\textsubscript{S574D} were described previously\textsuperscript{54}. TARDBP\textsubscript{WT}, TARDBP\textsubscript{S208S}, TARDBP\textsubscript{A315T}, and TARDBP\textsubscript{A327T} plasmids were gifts from Xinglong Wang. Mito-QC (pBabe.hygrom-cherry-GFP fia 101-152) was provided by Ian Ganley.

**RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR (qRT-PCR).** Total RNAs were extracted from patient fibroblasts using TRizol reagent (Thermo Fisher Scientific). Prior to reverse transcription, residual genomic DNA was removed with DNase I (Thermo Fisher Scientific). The cDNA was then reverse transcribed using transcription first-strand cDNA Synthesis Kit (Roche Applied Science) with 1 μg total RNA and oligo-dT as primer. All PCRs were performed in triplicate. qRT-PCR was carried out using SYBR Green Master Mix (Roche Applied Science) on a Light Cycler LC480. Results were normalized to β-2 macroglobulin or 28S genes. All primer sequences are included in Supplementary Table 2.

**Cell culture and transfection.** HeLa\textsubscript{YFP-Parkin}, HeLa with stable PINK1 expression (HeLa\textsubscript{PINK1-5S/His}), and a control host HeLa cell line were kind gifts from Richard Youle. PINK1\textsubscript{KD} HeLa cells and a matched control HeLa cell line were kindly provided by Wade Harper. HEK293T and SH-SY5Y cells were purchased from ATCC. Cells were maintained in culture in Dulbecco’s modified Eagle’s medium (Gibco) or DMEM/F12 50/50 (Corning) supplemented with 10% fetal bovine serum (Gibco), 1x penicillin/streptomycin (Invitrogen), and GlutaMax-1x (Gibco). Cells were transfected using FuGENE6 transfection reagent (Promega), Lipo-fectamine 3000 (Invitrogen), or jetPrime (Polyplus). RNAi-mediated knockdown of target genes was performed by transfection of the ON-TARGET plus-SMART pool siRNAs (Dharmacon) by using Lipofectamine RNAiMAX (Invitrogen) for the following genes: non-targeting control, PINK1, MFN1, MEN2, OPTN, NDP52, and DRP1. PINK1\textsubscript{490} and Ub\textsubscript{60} peptides were delivered by using PULSIn protein delivery reagent (Polyplus).

**CRISPR/Cas9-mediated gene editing and generation of cell lines.** Each two plasmid vectors (pSpCas9(BB)-2A-Puro (XP1992 V2.0) containing a single guide RNA (sgRNA) oligomer for CHCHD10 and CHCHD2 targeting near the N-terminal region of CHCHD10 and as follows: 5′-GGCTCCTGGGAAGCCG-CAGG-3′ and 5′-GCCGCGCACTCGCCGCA′-3′ and 5′- CTCAGATGAGAGCTGACCACC-C3′. The plasmids were transfected into HeLa and HeLa\textsubscript{YFP-Parkin} cells with jetPrime reagent. After 24 h, the transfected cells were detached with trypsin and plated individually into 96-well round-bottom plates. Cells were then expanded, and single clones were analyzed by immunofluorescence and immunoblotting to screen protein levels in CHCHD10 knockout and CHCHD10/2 double knockout.

**Antibodies and immunoblotting.** The following primary antibodies were used: FLAG (Sigma and Proteintech, 1:1000), HA (Cell Signaling and Proteintech, 1:1000), Myc (Proteintech, 1:1000), CHCHD10 (Proteintech, 1:1000), CHCHD2 (Proteintech, 1:1000), TOM20 (Cell Signaling and Santa Cruz Biotechnology, 1:1000), TDP43 (Proteintech and Santa Cruz Biotechnology, 1:1000), PINK1 (Santa Cruz Biotechnology and Novus, 1:1000), DRP1 (Cell Signaling Technology, 1:1000), MFN1 (Cell Signaling Technology, 1:1000), MFN2 (Cell Signaling Technology, 1:1000), NDP52 (Proteintech, 1:1000), and OPTN (Proteintech, 1:1000). Samples were collected and lysed in RIPA buffer (Cell Signaling Technology) containing a protease inhibitor cocktail (Sigma) and subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after measuring protein concentration by bicinchoninic acid (BCA) (Pierce). Immunoblots were visualized and analyzed with the Odyssey FC System (LI-COR).

**Co-immunoprecipitation.** HEK293T cells transfected FLAG-tagged CHCHD10 WT or S59L and TDP-43-tomato-HA or HA-tagged CHCHD10 WT or S59L and...
TDP-43-FLAG were solubilized with NP-40 lysis buffer (20 mM Tris, 137 mM NaCl, 1% NP-40, 2 mM EDTA with protein inhibitor cocktail). After sonication on ice, lysates were centrifuged. An equal amount of protein lysates were incubated with anti-FLAG M2 or anti-HA affinity gel (Sigma) overnight at 4°C washed three times with phosphate-buffered saline (PBS) containing 1% Tween-20, and then resuspended the pellet with 2× LDS sample buffer. The precipitates were subjected SDS-PAGE and analyzed with the Odyssey FC System (LI-COR).

Solubility and biochemical analyses. Transfected cells were washed twice with PBS, lysed in cold RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) and sonicated on ice. Cellular lysates cleared by ultracentrifugation at 100,000 × g for 30 min at 4°C to prepare RIPA-soluble fractions. RIPA-insoluble pellets were washed twice with protease inhibitors in cold PBS, sonicated, and re centrifuged. RIPA-insoluble proteins were extracted with urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris (pH 8.5)), sonicated, and centrifuged at 100,000 × g for 30 min at 22°C. Supernatants from the first centrifugation were analyzed for soluble fraction. Total protein in each sample was measured by the BCA method and resolved on ExpressPlus PAGE 4–20% gels (GenScript).

Cellular and mitochondrial fractionation. Mitochondria were isolated from cells with a Mitochondrial Isolation Kit (Thermo Scientific) by using Dounce homogenizers. A mixture of cytosolic and mitochondrial fractions was obtained after low-speed centrifugation at 500 × g for 15 min at 4°C. Mitochondrial-enriched pellets were collected at 3000 × g for 10 min at 4°C. Cytosolic supernatants were obtained and cleared by centrifugation at 12,000 × g for 10 min at 4°C, and contamination between fractions was analyzed with anti-tubulin and anti-HSP60 antibodies for each cytosolic and mitochondrial compartment.

Mitochondrial respiratory activity assay. Mitochondrial respiration in HeLa, CHCHD10KO HeLa, CHCHD2/10DKO HeLa, HeLaAFP, Park1, and CHCHD10KO HeLaAFP, Park1 cells were measured by using the Seahorse Extracellular Flux Analyzer XFp (Agilent Technologies) with the XF Cell Mito Stress Test Kit (Agilent Technologies). Transfected cells (1 × 10^6) were counted using ADAM-MC2 (NanoInstruments) and V3-96-well plates the day before performing the assay. Assay media were supplemented with 1 mM pyruvate, 2 mM glutamine, and 15 mM glucose. Standard mitochondrial stress tests were performed by first measuring basal values followed by measurements after sequential addition of 1 μM oligomycin, 0.5 μM FCCP, and 0.5 μM rotenone/antimycin A. After the assay, protein concentrations of each well were determined via BCA assay and used to normalize oxygen consumption rate values. The Seahorse assay parameters and experimental outcomes are summarized in Supplementary Table 3.

Immunofluorescence staining and imaging. The following primary antibodies were used: FLAG (Sigma and Proteintech, 1:200), FLAG-Alexa Fluor 488 (Invitrogen, 1:250), CHCHD10 (Proteintech, 1:200), CHCHD2/10 (Proteintech, 1:200), V5 (Life Technology, 1:200), TOM20 (Cell Signaling and Santa Cruz Biotechnology, 1:250), TDP-43 (Proteintech, 1:200), LAMP1 (Cell Signaling Technology, 1:200), and phosphorylated ubiquitin (BD Millennium). Samples were incubated on 4-well chamber slides (Lab-Tek), fixed with 4% paraformaldehyde in PBS (EMS Millipore), permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin (BSA) in PBS. Primary antibodies were diluted in 5% BSA in PBS and incubated overnight at 4°C. Samples were then rinsed three times with PBS-Tween 20 and incubated with secondary antibodies for 1.5 h at room temperature. Coverslips were mounted onto microscope slides with Prolong Diamond Antifade Reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). Samples were observed with an LSM 710 confocal microscope (Carl Zeiss) or Nikon Crest X-light c). Fly thoraxes from each group were collected and homogenized in 20 μl of homogenization buffer (100 mM Tris, 4 mM EDTA, and 6 M guanidine-HCl (pH 7.8)) and centrifuged at 16,000 × g for 10 min. Supernatants were diluted 1:200 and 1:10 with deionized water and subjected to ATP concentration and protein concentration measurements, respectively. ATP concentration was determined by using the CellTiter Glo Luminescent Cell Viability Assay Kit (Promega) and normalized to total protein.

Drosophila ATP assay. Fly thoraces from each group were collected and homogenized in 20 μl of homogenization buffer (100 mM Tris, 4 mM EDTA, and 6 M guanidine-HCl) and centrifuged at 16,000 × g for 10 min. The supernatants were diluted 1:200 and 1:10 with deionized water and subjected to ATP concentration and protein concentration measurements, respectively. ATP concentration was determined by using the CellTiter Glo Luminescent Cell Viability Assay Kit (Promega) and normalized to total protein.

Image analysis and statistical analysis. Mitochondrial branch length was measured by using the MIMA toolset combined with the ImageJ software.2 Statistical analysis was performed with Prism5 (GraphPad) software. For Drosophila eye quantification, we implemented ilastik, which is an interactive supervised machine learning-based tool for various bioimage analysis to classify ommatidia in fly eye images.3 Then we used Flynotyper, which is a computational tool for assessing the Drosophila eye morphological defects, to calculate phenotypic scores (P-scores), which indicate the irregularity of ommatidial arrangement. Eye images for group A (Fig. 1b, Supplementary Fig. 1d, Fig. 3a, Supplementary Fig. 3d, and Fig. 5a) were captured with a Leica M205C stereomicroscope equipped with a ring light and a Nikon SMZ1500 stereomicroscope (Nikon) equipped with a ring light and a Nikon DXM1200 digital camera. Although we applied the same criteria to analyze all eye images with ilastik and Flynotyper, the P-scores can be compared only within each experiment because of differences in the equipment, setting, and researchers. For statistical analyses of Seahorse Assays, Z-scores were calculated using OCR values from each plate. Z-score = (OCR value - mean of OCR values)/standard deviation of OCR values. Student’s t test (two-tailed), analysis of variance (ANOVA), and post hoc analysis (Tukey’s or Dunnett’s tests, two-tailed) were used to test statistical significance. All p values ≤0.05 were considered statistically significant.
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**Author contributions**
M.B., Y.-J.C., and N.C.K. conceived the project and performed experiments. S.B. performed all experiments using patient-derived fibroblasts. S.M. performed experiments measuring mitochondrial activity. M.B., Y.-J.C., S.B., S.M., J.H.K., V.P.-F., and N.C.K. analyzed data and wrote the manuscript. G.W.D. and J.P.T. provided guidance and helpful insights into design experiments and analyzed the data.

**Competing interests**
G.W.D. is the scientific founder of Mitochondria in Motion, Inc., which has license from WUSTL and is commercializing small-molecule mitofusin agonists for the treatment of neurodegenerative diseases. The remaining authors declare no competing interests.

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