The cellular stress proteins CHCHD10 and MNRR1 (CHCHD2): Partners in mitochondrial and nuclear function and dysfunction

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Coiled-coil-helix-coiled-coil-helix domain–containing 10 (CHCHD10) and CHCHD2 (MNRR1) are homologous proteins with 58% sequence identity and belong to the twin CX9C family of proteins that mediate cellular stress responses. Despite the identification of several neurodegeneration-associated mutations in the CHCHD10 gene, few studies have assessed its physiological role. Here, we investigated CHCHD10’s function as a regulator of oxidative phosphorylation in the mitochondria and the nucleus. We show that CHCHD10 copurifies with cytochrome c oxidase (COX) and up-regulates COX activity by serving as a scaffolding protein required for MNRR1 phosphorylation, mediated by ARG (ABL proto-oncogene 2, nonreceptor tyrosine kinase (ABL2)). The CHCHD10 gene was maximally transcribed in cultured cells at 8% oxygen, unlike MNRR1, which was maximally expressed at 4%, suggesting a fine-tuned oxygen-sensing system that adapts to the varying oxygen concentrations in the human body under physiological conditions. We show that nuclear CHCHD10 protein down-regulates the expression of genes harboring the oxygen-responsive element (ORE) in their promoters by interacting with and augmenting the activity of the largely uncharacterized transcriptional repressor CXXC finger protein 5 (CXXC5). We further show that two genetic CHCHD10 disease variants, G66V and P80L, in the mitochondria exhibit faulty interactions with MNRR1 and COX, reducing respiration and increasing reactive oxygen species (ROS), and in the nucleus abrogating transcriptional repression of ORE-containing genes. Our results reveal that CHCHD10 positively regulates mitochondrial respiration and contributes to transcriptional repression of ORE-containing genes in the nucleus, and that genetic CHCHD10 variants are impaired in these activities.

CHCHD10, although highly expressed in heart and skeletal muscle (7), was discovered to be mutated in a proportion of individuals suffering from several neurodegenerative diseases (8–17). The clinical effects of these mutations have been well described (18). Little is known, however, about the physiological role of CHCHD10 (7). Most studies have focused on characterizing CHCHD10’s effects in mitochondria by testing the effects of pathogenic mutations in a cell culture system (8, 9, 14). These studies have suggested that CHCHD10 affects stability of mitochondrial cristae (9, 14), COX activity (7), formation of mitochondrial networks (8), stability of mitochondrial DNA, and apoptosis (14).

Here, we demonstrate that CHCHD10, like MNRR1, is a hypoxia-responsive gene whose product functions in both the mitochondria and the nucleus. In the mitochondria, CHCHD10 interacts with COX and stimulates oxygen consumption. Furthermore, CHCHD10 regulates phosphorylation of MNRR1, thereby contributing to the role of MNRR1 in maintenance of cysteine residues separated usually by nine amino acids. These four cysteines form two disulfide bonds that stabilize the prototypical coiled-helix-coiled-helix-coiled-helix domain (CHCHD) that traps CHCHD10 and other twin CX9C family members in the mitochondrial intermembrane space (IMS)2. Other members of this family include CHCHD4 (also called Mia40), necessary for import and proper folding of twin CX9C proteins in the IMS (2), and CHCHD3, which acts with CHCHD6 to stabilize mitochondrial cristae (3, 4). Another recently characterized member of this family is MNRR1 (mitochondrial nuclear retrograde regulator 1, also called CHCHD2), a protein that is up-regulated under hypoxic stress, and maximally so at 4% oxygen. Besides residing in the mitochondria, MNRR1 is also present in the nucleus. Mitochondrial MNRR1 interacts with cytochrome c oxidase (COX) and is required for optimal enzyme function.

A stable knockdown of MNRR1 resembles a mitochondrial disease phenotype with decreased oxygen consumption, decreased cellular growth and mitochondrial membrane potential, and increased production of reactive oxygen species (ROS) (5). Nuclear MNRR1 regulates transcription of genes that harbor a conserved 13-bp sequence needed for the response to low oxygen levels that is termed the oxygen-responsive element (ORE) (6).

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2 The abbreviations used are: IMS, intermembrane space; ROS, reactive oxygen species; ORE, oxygen-responsive element; COX, cytochrome c oxidase; KD, knockdown; IP, immunoprecipitation.
electron transport chain function. However, unlike MNRR1, CHCHD10 in the nucleus functions as a transcriptional repressor of genes containing the ORE. To gain insight into CHCHD10’s normal and pathogenic roles, we tested two disease-associated point mutations (G66V and P80L) for nuclear and mitochondrial function. We find that both mutants fail to repress transcription at the ORE. The same mutants also are defective for regulation of oxygen consumption and increase production of mitochondrial ROS. The mitochondrial defects seen with the CHCHD10 mutants result from its reduced interaction with MNRR1.

We previously showed that MNRR1 is phosphorylated by ARG (Abl2 kinase), promoting binding to and activation of COX (22). We now show that CHCHD10 acts as a scaffolding protein, assisting ARG-mediated phosphorylation of MNRR1, and that this scaffolding does not occur when CHCHD10 harbors either the G66V or the P80L replacements, leading to decreased oxygen consumption and increased ROS levels. Our findings provide new mechanistic insights into the role of CHCHD10 under physiological conditions and a potential mechanism for the pathogenic effects seen in several mutant versions.

Results

CHCHD10 is a hypoxia-sensitive gene

Given the previous observations that MNRR1 production is maximally sensitive to 4% hypoxia (6), we tested the hypoxia sensitivity of CHCHD10 at a range of physiologically encountered oxygen tensions (19) between 1 and 13%. CHCHD10 is maximally up-regulated at 8% oxygen and down-regulated at 4% oxygen in a manner opposite to MNRR1, which is maximally induced at 4% oxygen and down-regulated at 8% and higher (Fig. 1A). The up-regulation of CHCHD10 at 8% is transcriptionally mediated (Fig. 1B). To confirm these effects, we analyzed CHCHD10 levels at 7, 8, and 9% oxygen. The maximally up-regulated CHCHD10 at 8% oxygen takes place in a surprisingly narrow window (Fig. 1C). Thus, different ratios of these two proteins are present at oxygen concentrations in the range analyzed.

CHCHD10 is localized to both the nucleus and the mitochondria

CHCHD10 has been characterized as a mitochondrial protein in previous studies (7, 9). Because of its high degree of conservation with MNRR1 (58% identical), a bi-organellar protein localized to both the mitochondria and the nucleus (6), we examined the subcellular localization of CHCHD10. Similar to MNRR1, CHCHD10 is also present in both the mitochondria (primarily) and the nucleus as analyzed by cell fractionation (Fig. 2A) and by confocal microscopy (Fig. 2B). The mitochondrial localization of CHCHD10 is well established (7, 9, 20). In the nucleus CHCHD10 colocalizes with DAPI as suggested by a positive Pearson correlation coefficient. A recent study also showed nuclear localization (21). We further validated the nuclear signal by use of a stable knockdown of CHCHD10 in HEK293 cells (Fig. 2B, top panel, far right). In these cells there is a clear reduction in the nuclear signal (Fig. 2B, top panel) compared with WT cells also at 20% oxygen (Fig. 2B, middle panel, bar graph to right of panel), suggesting the signal does not arise from antibody cross-reactivity. At 8% oxygen CHCHD10 is increased in both the nucleus and the mitochondria (Fig. 2B).

To gain further insight into CHCHD10’s role in vivo we tested cell growth of WT versus CHCHD10 knockdown (KD) cells in both glucose and galactose media. Growth of cells with glucose as the primary carbon source yielded indistinguishable growth rates independent of CHCHD10 level. However, when galactose was used as the primary carbon source, requiring cells to make ATP primarily through mitochondria (22), CHCHD10-KD cells grew more slowly than WT cells (Fig. 2C). The decrease in growth in galactose media was also seen by

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Figure 1. CHCHD10 is a hypoxia-sensitive gene. A, HeLa cells were incubated for 48 h at the stated O2 levels. Whole cell lysates were separated on an SDS-PAGE gel and analyzed for CHCHD10 and MNRR1 levels. GAPDH was probed as a loading control. B, real-time PCR analysis of transcript levels of endogenous CHCHD10 at 20, 8, and 4% oxygen (n = 4; *, p < 0.05). C, as in A but not probed for MNRR1. Error bars in graphs represent 1 standard deviation from the mean of repeat determinations.
counting live cell numbers (Fig. 2D). We also tested production of total cellular ROS using CM-H2DCFDA and found that CHCHD10-KD cells generate higher ROS levels (Fig. 2E). Increased ROS levels and slower growth specifically in galactose indicate defective oxidative phosphorylation.

CHCHD10 stimulates COX activity in the mitochondria

CHCHD10 has previously been characterized as a soluble protein in the mitochondrial IMS (9), and a transient knockdown of CHCHD10 decreased COX activity and ATP levels (7). Complementing these observations, we find that CHCHD10 copurifies with COX (Fig. 3A, upper left) and interacts with the intact complex in vivo (Fig. 3A, upper right). Moreover, CHCHD10 is defective for its interaction with COX in the absence of MNRR1 (Fig. 3A, lower). When we separated purified bovine heart COX on a high percentage urea-acrylamide gel that separates COX into its component subunits (23), we detected CHCHD10 immunologically, supporting its interaction with COX (Fig. 3B). The amount of CHCHD10 appears, however, to be submolar, similar to MNRR1 (Fig. 3B) (5). Inter-
Interestingly, when we added increasing amounts of recombinant CHCHD10 to purified COX in vitro we observed stimulation of COX activity (Fig. 3C). We then tested the effect of CHCHD10 on COX activity in vivo, by measuring intact cellular oxygen consumption. In WT cells, overexpression of CHCHD10 increases oxygen consumption (Fig. 3D) whereas in CHCHD10-KD cells oxygen consumption is decreased to ~60% that in WT cells. Overexpression of CHCHD10 in KD cells restores oxygen consumption. Note that the restoration is equivalent to WT cells overexpressing CHCHD10 (Fig. 3D). We then asked whether overexpression of WT-CHCHD10 can increase oxygen consumption in MNRR1-KO cells. We found that it could not
consistent with the requirement for MNRR1 for CHCHD10 to interact with COX (Fig. 3A, lower). We also examined oxygen consumption under 8% hypoxia in WT and CHCHD10-KD cells, which was increased by nearly 90% in WT cells and 40% in CHCHD10-KD cells (Fig. 3B, left). We reasoned that the increase in CHCHD10-KD cells may be because of up-regulation of CHCHD10 under hypoxia and examined protein levels in CHCHD10-KD cells at 8%. Using Western blot analysis we see that CHCHD10 levels are up-regulated in the CHCHD10-KD under hypoxia comparable to WT levels (Fig. 3C, right). A similar effect was seen on oxygen consumption. Under hypoxia, oxygen consumption in CHCHD10-KD cells increased to levels comparable to WT at 20%. Hence, at 8% hypoxia, the up-regulated CHCHD10 increases oxygen consumption rate. Taken together, the data show that CHCHD10 functions to stimulate COX activity under normoxia as well as hypoxia in the mitochondria and that the presence of MNRR1 is necessary for this stimulation.

Defective mitochondrial oxygen consumption in CHCHD10-KD cells arises via defective phosphorylation of MNRR1

To investigate the mechanism of decreased oxygen consumption in CHCHD10-KD cells, we assessed whether CHCHD10’s homolog MNRR1 can rescue the decreased oxygen consump-
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ntion. We found that transfecting increasing amounts of MNRR1 cannot increase oxygen consumption in CHCHD10-KD cells (Fig. 4A). Because mitochondrial stimulation of respiration by MNRR1 requires its phosphorylation on Tyr-99 by Abl2 kinase/ARG (24), we examined the phosphorylation level of endogenous MNRR1. Despite elevated levels of MNRR1 in the mitochondria, MNRR1 is less phosphorylated in CHCHD10-KD than in WT cells (Fig. 4B). Furthermore, phosphorylation of overexpressed mitochondrial MNRR1 was reduced (Fig. 4C).

To investigate whether ARG is responsible for reduced MNRR1 phosphorylation, we examined levels of ARG in CHCHD10-KD mitochondria and found them to be lower than WT levels (Fig. 4C).

We hypothesized from the above observations that reduced ARG in mitochondria caused reduced MNRR1 phosphorylation, and thus reduced interaction with COX and decreased oxygen consumption, thereby rationalizing the inability of the overexpressed MNRR1 to suppress the oxygen consumption defect in CHCHD10-KD cells (Fig. 4A). To test this hypothesis, we overexpressed phosphomimetic MNRR1 (Y99E), which is expected to circumvent the reduced MNRR1 phosphorylation seen in CHCHD10-KD cells. We indeed observed rescue of oxygen consumption in CHCHD10-KD cells (Fig. 4D) whereas we did not see this rescue when we overexpressed WT or non-phosphorylatable (Y99F) MNRR1 at the same level. We can also see this effect in vitro using purified components. Maximum COX stimulation is seen with a combination of phosphomimetic MNRR1 and CHCHD10 as compared with each by itself (Fig. 4E).

In the nucleus CHCHD10 functions as a repressor at the ORE

The presence of CHCHD10 in both the nucleus and the mitochondria suggests that CHCHD10, like MNRR1, plays a role in both organelles. To characterize the effects of CHCHD10 in the nucleus, we used a luciferase reporter carrying the COX4I2 promoter, which harbors the 13-bp ORE (25).

Unlike MNRR1 (6), overexpression of CHCHD10 inhibits transcription of the COX4I2 reporter; this inhibition requires an intact ORE (6) (Fig. 5A). We further investigated this inhibition in CHCHD10-KD cells with reporters harboring both a COX4I2 ORE (Fig. 5B) and an MNRR1 ORE (Fig. 5C). These results suggest that both COX4I2 and MNRR1 are transcriptionally up-regulated in CHCHD10-KD cells. We also examined reporter activity at 8% oxygen and found that transcription of the COX4I2 reporter is repressed at 8% oxygen to ~60% of transcription at 20% oxygen (Fig. 5D). This is comparable to the effect seen by overexpressing WT-CHCHD10 under normoxia.

Transcription at the ORE was previously shown to be governed by three proteins: RBPjks, CXXC5, and MNRR1 (6). At 20% oxygen, CXXC5 interacts with RBPjk at the ORE to repress transcription, whereas at 4% MNRR1 displaces the inhibitory CXXC5 and activates RBPjk-mediated transcription at the ORE (6) (see Fig. 7B). Because CHCHD10 function requires an intact ORE, we hypothesized that CHCHD10 interacts with CXXC5 to strengthen repression at the ORE and tested this hypothesis by examining the interaction of CHCHD10 with CXXC5. We found that CHCHD10 co-immunoprecipitates with CXXC5 and vice versa (Fig. 5E). This presence of CXXC5 is essential for CHCHD10 to interact with the ORE because transient knockdown of CXXC5 prevents the interaction of CHCHD10 with the COX4I2 ORE (Fig. 5F).

These results suggest that CXXC5 is necessary for CHCHD10 to function at the ORE. Furthermore, CHCHD10 does not interact with MNRR1 in the nucleus (Fig. 5G), consistent with opposed functions of CHCHD10 and MNRR1 in this cellular compartment.

Point mutations in CHCHD10 abrogate its function in both the nucleus and the mitochondria

More than 10 different mutations in CHCHD10 have been linked with neurodegenerative diseases, and most lie within the region encoded by exon 2 (amino acids 15–87) (26). Two functionally uncharacterized mutations found in this region, G66V and P80L, were chosen for study because they have been associated with several different phenotypes including ALS (9, 12), Charcot-Marie-Tooth neuropathy type 2A (CMT2A) (27), and spino muscular atrophy (15). In fact, the G66V mutation has been associated with a spectrum of phenotypes within the same family (28). Importantly, unlike some mutations, these have not been identified in any control population in several different studies (29), strengthening their disease association. To investigate the nuclear function of these mutants, we examined their ability to inhibit the COX4I2 reporter. We found that overexpression of these CHCHD10 mutants, unlike WT, failed to repress transcription of the COX4I2 reporter (Fig. 6A). We also examined these effects at the translational level by analyzing levels of COX4I2 and MNRR1, two ORE-regulated proteins. Both are repressed by WT-CHCHD10 but not by the mutants (Fig. 6B). To identify the mechanism for the failure to repress, we compared the interaction of WT-CHCHD10 and the point mutants with ORE inhibitor protein CXXC5. Both mutant CHCHD10 proteins were found to be defective for interaction with CXXC5 (Fig. 6C).

Because CHCHD10 interacts with COX in the mitochondria and promotes its activity, we examined whether either of the two mutations could rescue the oxygen consumption defect seen in CHCHD10-KD cells. We found that, unlike WT-CHCHD10, neither point mutant was able to rescue these defects, being no better than an overexpressed empty vector (Fig. 6D). We also tested the mitochondrial membrane potential in CHCHD10-KD cells and found it to be ~50% of WT cells. Overexpression of WT-CHCHD10 can rescue this defect whereas neither point mutant was able to rescue the defective membrane potential (Fig. 6E). Moreover, each mutant increased production of mitochondrial ROS, in contrast to WT-CHCHD10, which decreased ROS production (Fig. 6F). To examine the mechanism by which this could occur, we tested for the interaction of WT or mutant CHCHD10 with MNRR1 and COX. Each mutant protein was defective for binding to MNRR1 and thereby COX (Fig. 6G). A failure to bind MNRR1 leads to reduced ARG-mediated phosphorylation of MNRR1, which then cannot bind to and stimulate COX activity. Hence, the G66V and P80L mutant CHCHD10 proteins may be defective for stimulating mitochondrial oxygen consumption, presumably leading to production of ROS.
Discussion

Mitochondrial energy production is regulated to respond to extracellular changes, a key feature of cellular homeostasis that requires mitonuclear signaling to alter gene transcription (30, 31). We previously showed that MNRR1 is a component of a mitonuclear pathway whose signaling is altered under oxidative stress (5, 6, 24) and we now show that it functions in coordination with CHCHD10. CHCHD10 and MNRR1 belong to the twin CX9C family of proteins that primarily form scaffolds for the formation and the organization of the large macromolecular complexes found in mitochondria (32) such as cytochrome c oxidase, a complex composed of 14 subunits (33). MNRR1 and CHCHD10 are highly conserved (58% in humans); have a common homolog in yeast, Mix17; and appear to be the product of a gene duplication event prior to the mammalian radiation. Moreover, the presence of disulfide linkages to stabilize the
Figure 6. Point mutations in CHCHD10 abrogate CHCHD10’s function in the nucleus and the mitochondria. A, top, HEK293 cells were cotransfected with 100 ng COX4I2-luciferase WT or mutant reporter plasmid and either 200 ng empty vector (EV) or the different CHCHD10 expression plasmids. After 48 h, a dual luciferase assay was performed to measure reporter activity (n = 4; *, p < 0.05). Bottom, equal amounts of nuclear fractions transfected with empty vector (EV), WT-CHCHD10, or point mutants were separated on an SDS-PAGE gel and probed with CHCHD10. DRBP76 was probed as a loading control and mitochondrial protein NDUFS3 monitored fractionation. B, equal amounts of whole cell lysates transfected with empty vector (EV), or with WT or point mutants of CHCHD10, were separated on an SDS-PAGE gel and probed for MNRR1 and CHCHD10. GAPDH was used as a loading control. C, HEK293 cells were cotransfected with CXXC5 and with WT or CHCHD10 (FLAG-tagged) point mutants. After 48 h nuclear lysates were immunoprecipitated with CXXC5-conjugated beads and probed for FLAG-tagged CHCHD10. Equal amounts of input fractions were also probed for FLAG and CXXC5. D, above, WT cells were transfected with an empty vector as a control and CHCHD10-KD cells were transfected with either empty vector (EV), WT, or point mutants for CHCHD10. Oxygen consumption was measured after 24 h (n = 4; *, p < 0.05). Below, equal amounts of whole cell lysates of WT cells transfected with an empty vector and CHCHD10-KD cells transfected with empty vector (EV), WT-CHCHD10, or point mutants were separated on an SDS-PAGE gel and probed with CHCHD10. GAPDH served as a loading control. E, WT cells were transfected with an empty vector as a control and CHCHD10-KD cells were transfected with either empty vector (EV), WT, or point mutants for CHCHD10. After 24 h, 40,000 cells/well were plated on a 96-well plate and mitochondrial membrane potential was measured using TMRM (Invitrogen) per the manufacturer’s protocol (n = 4; *, p < 0.05). Results are shown as fluorescence relative to the empty vector sample.
CHCH domain imparts potential redox sensitivity to these proteins, which may be essential to their ability to regulate the stability of COX under altered oxygen tensions (32). The fact that MNRR1 and CHCHD10 function as a complex was also recognized in a recent analysis of an ALS-associated mutation, R15L, in CHCHD10 (20).

CHCHD10 and MNRR1 in the mitochondria bind to COX and regulate its activity. MNRR1’s mitochondrial role has been characterized previously (5), including the key step of its phosphorylation by Abl2 kinase/ARG (24). In the present study, we identified the mitochondrial role of CHCHD10 to recruit ARG to phosphorylate MNRR1. This phosphorylation enhances the binding of MNRR1 to COX and increases COX activity. The above role for CHCHD10 is supported by the observations of reduced phosphorylation of MNRR1 and decreased oxygen consumption in CHCHD10-KD cells. Importantly, only a phosphomimetic version of MNRR1, but not WT-MNRR1, can rescue the respiration defect in CHCHD10-KD cells (Fig. 4D).

MNRR1 and CHCHD10 are also present in the nucleus, where they positively and negatively regulate transcription of ORE-harboring genes, respectively. Moreover, both proteins are oxygen regulated, induced maximally at two distinct oxygen tensions, 4% for MNRR1 (6) and 8% for CHCHD10 (Fig. 1). Thus, the relative effect of each on transcription of ORE-containing genes may change continuously as oxygen levels decline. Experimental oxygen tension is 20%, although this is high compared with physiological levels in vivo, which range from ~3 to 8% in most tissues and ~13% in lung (19, 34). One of the most critical periods of oxygen sensing is during placental development when oxygen levels switch from 2 to 3% prior to 12 weeks of gestation to 8% afterward (35). Different oxygen levels likely require the regulation of factors that respond appropriately and alter transcription. The DNA element in the CHCHD10 promoter that responds maximally at 8% oxygen is yet to be characterized. Furthermore, other cellular factors besides MNRR1 and CHCHD10 and other oxygen-responsive elements may be responsible for regulating mitochondrial function at different oxygen tensions. The twin Cx3C protein CHCHD3 is also known to regulate transcription, at the BAG1 promoter (36), although its hypoxia sensitivity is yet to be tested.

Mutations in CHCHD10 have been associated with a number of neurodegenerative diseases. Several studies have characterized the effects of CHCHD10 by analyzing these mutations in skin fibroblasts from patients (9, 14, 20) or by overexpression of these mutations in WT cells (8, 37). Here we show that CHCHD10 plays a key role in the regulation of electron transport chain function in the mitochondria as well as a novel role in the nucleus as a transcriptional repressor at the ORE. We selected two CHCHD10 mutations, P80L and G66V, for detailed study. These mutations are associated with several phenotypes across different populations and are absent in control populations. In the mitochondria, each mutant decreased mitochondrial oxygen consumption and increased production of mitochondrial ROS. High ROS levels have been extensively associated with neurodegeneration and specifically are consistent with neurodegenerative phenotypes such as ALS (38); motor neuron disease, in which ROS may affect cross talk between neurons and muscles (39); and Parkinson’s disease (40). Unlike WT-CHCHD10, which is necessary to recruit ARG to phosphorylate MNRR1 (Fig. 4, B and C), the mutants do not interact with MNRR1 (Fig. 6G) and they may not recruit ARG to phosphorylate MNRR1. MNRR1 phosphorylation by ARG is essential to its role in regulation of COX activity, and a mutant version of MNRR1 that has defective phosphorylation has been linked to another neurodegenerative disorder, Charcot-Marie-Tooth disease type 1A (24). Thus, even in the presence of elevated levels of MNRR1 the two mutant versions of CHCHD10 prevent phosphorylation of MNRR1, giving rise to the mitochondrial phenotype seen. A recent study also showed that CHCHD10 is localized to both the mitochondria and the nucleus and that its normal function prevents the neurotoxic mitochondrial localization of the protein TDP-43 whereas two other disease-associated mutations, R15L and S59L, promote its cytoplasmic mislocalization (21). These mutations have also been shown to accumulate excessive mitochondrial iron (37).

A feature of the pathogenic mutations not well explained thus far is their generally heterozygous appearance, suggesting they are dominant mutations, whereas in cultured cells the two mutations examined do not appear dominant. Despite being overexpressed, endogenous expression needs to be reduced to see a clear phenotype. An important caveat, however, is that the neurodegenerative diseases to which these mutations have been linked show late onset. Consequently, these experiments do not preclude a small degree of haploinsufficiency that, over time, leads to the observed pathophysiologies. Because there is evidence of population-specific effects of some of the mutations (41–43), it is also possible that other variants must be present to elicit a full phenotype, a possibility also suggested by the diversity of neurodegenerative diseases in which mutations in CHCHD10 have been found.
CHCHD10 and MNRR1 in mitonuclear function and dysfunction

In summary, we have characterized the role of CHCHD10 as a key modulator of mitochondrial function via interorganellar signaling (Fig. 7). The failure of several pathogenic mutants, which no longer interact with COX, to rescue the mitochondrial effects of CHCHD10 depletion provides a potential mechanism for CHCHD10 dysfunction during the pathogenesis of neurodegenerative diseases. In addition, the altered transcriptional regulation in the nucleus of these mutants at ORE-harboring promoters provides a further pathogenic pathway. Lastly, the hypoxia sensitivity of both CHCHD10 and MNRR1 transcription places the CHCHD10-MNRR1 pair in a regulatory network that can respond to altered oxygen levels. Such altered levels may be present in different tissues, during different developmental stages, or as a result of disease, and whose lack may therefore contribute to neurodegenerative disease phenotypes with which CHCHD10 mutations have been associated.

Experimental procedures

Cell culture

Experiments were performed with HEK293 cells and HeLa (human cervical cancer) cells. The HEK293 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan, UT) with 10% FBS (Sigma-Aldrich) plus antibiotic and antimitotic (HyClone); CHCHD10 knockdown (sc-72754, Santa Cruz Biotechnology (SCBT), Dallas, TX) cells were grown under puromycin selection. HEK293 cells stably overexpressing WT or mutant CHCHD10 were selected using G418.

The CHCHD10 shRNA is a pool of two different shRNA plasmids: hairpin sequence GATCCGTGCAAGTACTACCACTGGTTTCAAGAGACACCATTGATGACTTCGACCTTTTTT and corresponding siRNA sequences (sc-72754A) (sense, GUGCAAGUACUACCAGUUGTT; antisense, ACCAUGGUAGUACUGUCAGTT) and the sc-72754B hairpin sequence GATCCCAAGCACAAACAAGAGATTTTCAAGAGAATCTGGTGTGTTGTGCTTGGT and corresponding siRNA sequences (sc-72754B) (sense, CAGACCCAAACAACCAUTT; antisense, AAUCUGGUGUUGGUGUCUGTT). Note that all sequences are provided in 5′ → 3′ orientation.

Effector and reporter plasmids

The WT and mutant 579-bp COX4I2 promoter luciferase reporter plasmids (6) and MRNR1 reporter plasmid (5) have been described previously. The WT MRNR1 and CHCHD10 expression plasmid was cloned in the pCI-Neo vector with a C-terminal 3× FLAG epitope. The G66V and P80L mutants were cloned in the pCI-Neo vector using overlap extension PCR. The mutation was confirmed by sequencing. All the expression plasmids were purified using the EndoFree plasmid purification kit (Qiagen, Valencia, CA). CXXC5 was expressed from a pCI-Neo vector.

Transient transfection of HEK293 cells

HEK293 cells were transfected with the indicated plasmids using TransFast transfection reagent (Promega, Madison, WI) according to the manufacturer’s protocol. A TransFast:DNA ratio of 3:1 in serum- and antibiotic-free medium was used. Following incubation at room temperature for ~15 min, the cells were overlaid with the mixture. The plates were incubated for 1 h at 37 °C followed by replacement with complete medium and further incubation for the indicated time interval.

Real-time PCR

Total cellular RNA was extracted using an RNeasy Plus Mini Kit (Qiagen) per the manufacturer’s instructions. Complementary DNA (cDNA) was generated by reverse transcriptase PCR using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). Transcript levels were measured by real time PCR using SYBR Green on an ABI7500 system. Real-time analysis was performed by the ΔΔCt method (44). The primer sequences used were as follows: CHCHD10 (forward, CGCCTACGAGATCAGGCAG; reverse, GGAGCTCAGACCATGTTGTACTTTG) and 18S rRNA (forward, AGTCCCTGGCCTTTTGTACACA; reverse, GATCCGAGGGCCTCACTAAAC).

Hypoxia assays

Cells were maintained either at 20% oxygen (normoxia) or at 13, 9, 8, 7, 4, or 1% oxygen in a chamber infused with N2 and CO2 at 37 °C. The gas flow was controlled with PRO-OX 110 and PRO-CO2 controllers (BioSpherix, Redfield, NY) to achieve desired oxygen levels and 5% CO2. Oxygen equilibration time (3–4 h) was accounted for in the hypoxia experiments.

Luciferase reporter assays

Luciferase assays were performed using the dual-luciferase reporter assay kit (Promega) per the manufacturer’s instructions. Transfection efficiency was normalized with the cotransfected pRL-SV40 Renilla luciferase expression plasmid.

DNA-binding assays

DNA-binding assays were performed (6) with a few modifications. HEK293 WT (scrambled) or CXXC5 KD (siRNA sc-91677 from SCBT) was transfected with a CHCHD10 expression plasmid. For sc-91677, CXXC5 siRNA is a pool of three different siRNA duplexes: sc-91677A (sense, CUCUCUCCUACUCCUCUUCUTT; antisense, UCAUAAUGAAGGCUUGT); sc-91677B (sense, CCAGGCCUCUCAUUGATT; antisense, UUAUAGCGGAGGAGGAGT); sc-91677C (sense, CGACUUGUGAGAUCGAATT; antisense, UUUUGAACUCAACACCGT). All sequences are provided in 5′ → 3′ orientation.

After 48 h proteins were cross-linked and nuclei were separated. DNA was fragmented by incubation with Sau3AI and nuclei were lysed. The antibody-matrix complex was prepared as described under “Immunoblotting and co-immunoprecipitation (IP)” using anti-CHCHD10 antibody and shaken overnight with nuclear lysate at 4 °C. Cross-linking was reversed and the precipitated DNA was purified with the QIAquick purification kit (Qiagen) and amplified with primers encompassing the segment harboring the endogenous ORE for both the CHCHD10 and IgG IP samples.

Cell proliferation assay

Cell proliferation was assessed with the CellTiter 96 AQueous One colorimetric solution kit (Promega) in a 96-well plate.
according to the manufacturer’s instructions. Equal numbers of cells were plated and analyzed for proliferation at 24 h (~1.5 doublings) and 48 h (~3 doublings) by treating with CellTiter 96 aqueous solution and recording absorbance at 490 nm.

**Cell counting assay**

Live cell counts were performed by trypan blue staining on a Countess™ automated cell counter (Invitrogen). Live cells (7.5 × 10⁶) were plated and allowed to grow for 48 h (~3 doublings), and the number of live cells counted again.

**Intact cellular oxygen consumption**

Cellular oxygen consumption was measured with a Seahorse XFe24 Bioanalyzer. Cells were plated at a concentration of 40,000 per well one day prior to basal oxygen consumption measurements, performed according to the manufacturer’s instructions.

For the hypoxia experiments oxygen consumption was analyzed using the Oxygen Consumption Rate Assay Kit (Cayman, Ann Arbor, MI). Cells were plated at a concentration of 30,000 per well one day prior to measurements, incubated at 20 or 8% oxygen, and measurements were performed according to the manufacturer’s instructions.

**Cytochrome c oxidase assay**

*In vitro* COX activity was measured using the Cytochrome c Oxidase Assay Kit from Sigma-Aldrich as per manufacturer’s instructions. The cytochrome c oxidase used was purified from bovine heart cytochrome c. The cytochrome c oxidase assay was performed on bovine heart and dialyzed before being used for the assay.

**ROS measurements**

Total cellular ROS measurements were performed with CM-H₂DCFDA (Life Technologies). Cells were distributed into 12-well plates at 10⁵ cells per well and incubated for 24 h. Cells were then treated with 10 μM CM-H₂DCFDA in serum- and antibiotic-free medium for 1 h. Cells were washed twice in PBS and analyzed for fluorescence on a Gen5 Microplate Reader (BioTek Inc, Winooski, VT). For mitochondrial ROS measurements, the cells were treated as above but with 5 μM MitoSOX Red (Life Technologies) for 30 min.

**Confocal microscopy**

Confocal microscopy was performed as described (24). Primary antibodies were CHCHD10 (1:50, Sigma) and COXI (1:100, Life Technologies). Secondary antibodies were goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 596 (1:300, The Jackson Laboratory, Bar Harbor, ME). Cells were imaged with a Leica TCS S5P microscope and images were combined in Photoshop. Colocalization (overlap of the two fluorophores) and intensity (number of pixels per unit area) were quantitated using Volocity image analysis software (PerkinElmer Life Sciences). The Pearson correlation coefficient (24) in Fig. 2B is indicated on the images.

**Immunoblotting and co-immunoprecipitation (IP)**

Immunoblotting on a PVDF membrane was performed as described previously (5, 6). Unless specified otherwise, primary antibodies were used at a concentration of 1:500 and secondary antibodies at a concentration of 1:5000. The MNRR1 (19424–1-AP), NDUSF3 (15066–1-AP), DRBP76 (19887–1-AP), COX6B1(11425–1-AP), COX4 (11242–1-AP), GADPH (HRP-60004), and tubulin (HRP-66031) antibodies were purchased from Proteintech (Chicago, IL). The anti-CHCHD10 (HPA003440) and anti-FLAG HRP (A8592) conjugated antibodies were purchased from Sigma-Aldrich and the COX412 (H00084701-M01) antibody was purchased from Abnova Corp. (Taipei City, Taiwan). The TOM20 (72610) antibody was purchased from Cell Signaling Technology (Beverly, MA), and the anti-phosphotyrosine (4G10) antibody (05–321) was purchased from EMD Millipore (Burlington, MA). Co-immunoprecipitation experiments were performed per the supplier’s protocol by incubating the antibody-adsorbed beads overnight at 4 °C. For co-immunoprecipitation of FLAG-tagged proteins, Anti-FLAG M2 affinity gel (A220, Sigma-Aldrich) and anti-phosphotyrosine AC beads (sc-7020, SCBT) were used. The ARG antibody was a gift from Dr. Anthony Koleske (Yale University). The high percentage urea-acrylamide gel was run as described previously (23).

**Mitochondria isolation**

Mitochondria were isolated from cells with a Mitochondrial Isolation Kit (Thermo Scientific) according to the manufacturer’s protocol. The nuclear fraction was obtained by low-speed centrifugation and the mitochondrial fraction was obtained after high-speed centrifugation of the nuclear supernatant. Cross-contamination between the fractions was analyzed with compartment-specific antibodies.

**Statistical analysis**

Statistical analyses were performed with MSTAT version 6.1.1 (N. Drinkwater, University of Wisconsin, Madison, WI). The two-sided Wilcoxon rank sum test was applied to determine statistical significance for *p* values. Data were considered statistically significant with *p* < 0.05.

**Data availability**

All data generated or analyzed during this study are included in this published article.

**Author contributions**—N. P., M. S., L. I. G., and S. A. data curation; L. I. G. and M. H. funding acquisition; N. P. and M. S. validation; N. P., M. S., L. I. G., and S. A. investigation; N. P. and M. S. writing-original draft; N. P., M. S., M. H., L. I. G., and S. A. writing-review and editing; L. I. G. and M. H. resources; M. H. methodology; L. I. G. and S. A. conceptualization; L. I. G. and S. A. supervision; L. I. G. and S. A. project administration.

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