Mitochondrial disulfide relay mediates translocation of p53 and partitions its subcellular activity

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p53, a critical tumor suppressor, regulates mitochondrial respiration, but how a nuclear protein can orchestrate the function of an organelle encoded by two separate genomes, both of which require p53 for their integrity, remains unclear. Here we report that the mammalian homolog of the yeast mitochondrial disulfide relay protein Mia40 (CHCHD4) is necessary for the respiratory-dependent translocation of p53 into the mitochondria. In the setting of oxidative stress, increased CHCHD4 expression partitions p53 into the mitochondria and protects its genomic integrity while decreasing p53 nuclear localization and transcriptional activity. Conversely, decreased CHCHD4 expression prevents the mitochondrial translocation of p53 while augmenting its nuclear localization and activity. Thus, the mitochondrial disulfide relay system allows p53 to regulate two spatially segregated genomes depending on oxidative metabolic activity.

mitochondrial DNA | DNA repair | mutant p53

Along with regulating gene transcription in the nucleus, p53 protein also functions in the cytoplasm to regulate such cellular processes as apoptosis and autophagy (1, 2). p53 can bind to the outer membrane of the mitochondria by forming complexes with BclXL and Bcl proteins in response to apoptotic stimulus (3), and also can accumulate in the matrix and interact with cyclophilin D to open the mitochondrial permeability transition pore to trigger necrosis (4). Although best known as the "guardian of the genome," p53 also has been shown to be important for the repair and maintenance of mitochondrial DNA (mtDNA) (5–10). Cellular stressors, such as reactive oxygen species or even acute exercise, can promote p53 translocation into mitochondria and its interaction with matrix proteins polymerase-γ, mitochondrial transcription factor A, and superoxide dismutase 2 (7, 11, 12). Despite major advances in the field of mitochondrial protein import (13), whether the transfer of p53 through the intermembrane space and the highly charged electrochemical gradient of the inner membrane in normal cellular state occur passively or through an active mechanism with the potential for functional homeostatic regulation, remains largely unexplored.

Recent studies have unveiled a disulfide relay system consisting of the import receptor CHCHD4 and the FAD-dependent sulfhydryl oxidase (GFER) in the intermembrane space that mediates protein translocation into mitochondria (14, 15). Through its N-terminal Cys-Pro-Cys (CPC) motif, CHCHD4 forms a transient intermolecular disulfide bond with cysteine residues in substrate proteins targeted for translocation into the mitochondria (15). Of note, previous studies have shown that the structure and function of p53 is redox-sensitive, and that the oxidation of cysteines in p53 affects its subcellular distribution (16, 17). Under conditions of increased oxidative stress, p53 has two cysteine pairs (C135-C141 and C275-C277) that are capable of forming intramolecular disulfide bonds, a structural feature reportedly present in the typical CHCHD4 substrate (15, 18, 19). Given the sensitivity of p53 to the cellular redox state and its critical role in regulating redox homeostasis (17, 20), we examined whether the importation of p53 into mitochondria could be mediated by the CHCHD4 disulfide relay system.

Results

p53 Interacts and Colocalizes with CHCHD4 in Respiring Mitochondria. Compared with control lentiviral transduction, the stable overexpression of CHCHD4 in HCT116 cells, a human colon cancer cell line with endogenous WT p53, markedly increased the level of p53 in the mitochondrial fraction (Fig. 1*A*, lanes 3 and 4). Conversely, the stable knockdown of CHCHD4 using shRNA decreased the relative level of p53 in the mitochondrial fraction (Fig. 1*A*, lanes 7 and 8). The results of these subcellular fractionation experiments were confirmed using confocal immunofluorescent microscopy to show that overexpression of CHCHD4 increased the mitochondrial colocalization of p53 and CHCHD4, as indicated by the white color on merging with MitoTracker, whereas knockdown of CHCHD4 resulted in loss of the colocalization signal (Fig. 1*B*, merge all three). Similar subcellular localization of p53 mediated by CHCHD4 was also observed in primary human myoblasts, indicating that this phenomenon is applicable to other cell types as well (Fig. S1*A*).

The mitochondrial disulfide relay system requires the regeneration of oxidized CHCHD4 via its oxidase GFER, which in turn transfers its electrons to oxidized cytochrome *c*. Thus, the importation of proteins through this system requires active respiration for the reoxidation of reduced cytochrome *c* by respiratory complex IV (21). We previously created a nonrespiring HCT116 line by disrupting both copies of *Synthesis of Cytochrome c Oxidase 2* ($SCO2^{-/-}$), a gene essential for complex IV assembly (22, 23). To test whether the p53–CHCHD4 interaction depends on active respiration, we transiently coexpressed p53 with polyhistidine-tagged CHCHD4 (CHCHD4-His_6) in isogenic HCT116 cells with ($^{+/+}$) or without ($^{-/-}$) SCO2. Western blot analysis of

Significance

p53 is one of the most highly studied proteins in biomedical research because of its importance in preventing cancer and its direct or indirect role in many biological processes. It is best known as a nuclear protein that is critical for maintaining genomic integrity and regulating gene expression. We have uncovered a molecular mechanism by which p53 translocates into the mitochondria, depending on respiration, and facilitates the repair of oxidative damage to mitochondrial DNA. The dynamic partitioning of p53 between the nuclear and mitochondrial compartments has important implications for cancer and the many other essential functions of p53 in normal physiology.

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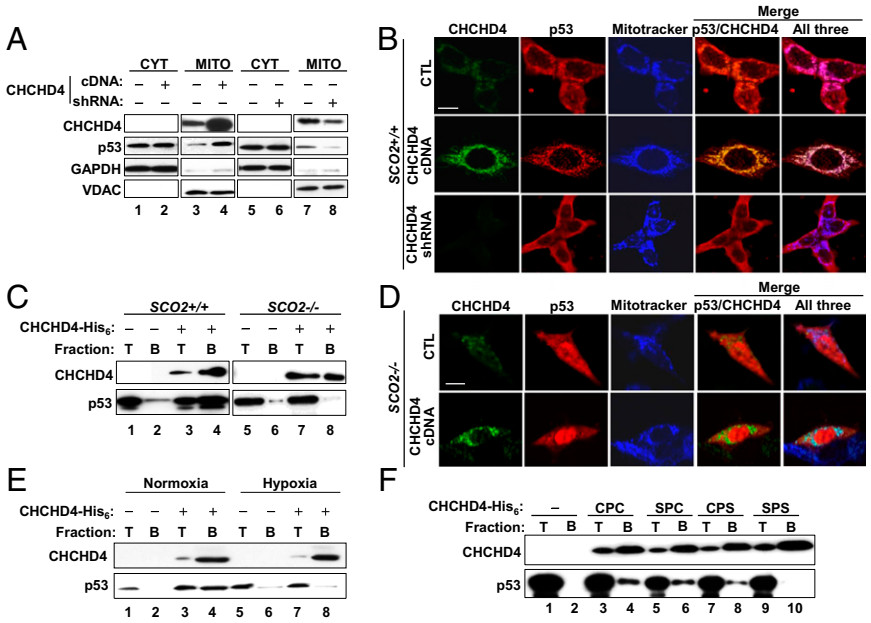

Fig. 1. p53 interacts and colocalizes with CHCHD4 in mitochondria. (*A*) Western blots of the cytosolic (CYT) and mitochondrial (MITO) fractions of WT HCT116 cells transduced with CHCHD4 cDNA or shRNA lentivirus versus control lentivirus (–) and then transfected with p53. GAPDH and VDAC serve as cyoplasmic and mitochondrial protein loading controls, respectively. (*B*) Confocal immunofluorescent localization of CHCHD4 (green) and p53 (red) in $SCO2^{+/+}$ (WT) cells transduced with CHCHD4 cDNA or shRNA lentivirus and then transfected with p53. MitoTracker (blue) indicates mitochondria. (*C*) Respiring ($SCO2^{+/+}$) and nonrespiring ($SCO2^{-/-}$) cells were cotransfected with CHCHD4-His_6 and p53, and their lysates were passed over Ni-NTA columns. Total lysate (T) and column-bound (B) fractions were subjected to Western blot analysis. (*D*) Immunofluorescent imaging of CHCHD4 (green), p53 (red), and mitochondria (MitoTracker, blue) in $SCO2^{-/-}$ cells. (*E*) Effect of hypoxia (1.5% O_2 for 48 h) on the interaction of cotransfected p53 and CHCHD4-His_6 by Ni-NTA binding assay in WT cells. (*F*) p53 interaction with the Cys-Pro-Cys (CPC) motif of CHCHD4 was tested by substituting Cys (C) with Ser (S). (Scale bars: 10 μm.)

the total (T) cell lysate compared with the nickel-NTA (Ni-NTA) column-bound (B) fraction of CHCHD4-His_6–expressing cells revealed a p53–CHCHD4 interaction in the respiring $SCO2^{+/+}$ cell line that was essentially undetectable in nonrespiring $SCO2^{-/-}$ cells (Fig. 1*C*, lanes 4 and 8). Confocal immunofluorescent microscopy showed increased accumulation of p53 in the nucleus of $SCO2^{-/-}$ cells, consistent with our previous report of elevated oxidative stress and p53 stabilization in these nonrespiring cells (control in Fig. 1*D*) (24).

Consistent with the protein–protein interaction data, overexpression of CHCHD4 failed to drive p53 into the mitochondria of the nonrespiring $SCO2^{-/-}$ cell, as indicated by the absence of either yellow (Fig. 1*D*, merge p53/CHCHD4) or white color (merge all three). Moreover, decreasing respiration by placing $SCO2^{+/+}$ cells in hypoxia disrupted the p53–CHCHD4 interaction (Fig. 1*E*, lane 4 vs. lane 8). At the structural level, the specificity of this interaction was further confirmed by substituting the two cysteine residues in the CPC motif of CHCHD4 with serine, which abolished p53 binding (Fig. 1*F*, lane 10).

CHCHD4 Regulates p53 Nuclear Localization and Activity. Oxidative stress causes p53 stabilization and accumulation in the nucleus, with subsequent transactivation of its target genes. We hypothesized that modulating the levels of CHCHD4 via cDNA expression or shRNA-mediated knockdown (total cell lysates, Fig. 2*A*) would shift the distribution of p53 and affect its activity in the nucleus. The treatment of WT HCT116 cells with H_2O_2 increased the level of p53 in the nucleus, but, as predicted, this increase was attenuated by concomitant expression of CHCHD4 (Fig. 2*A*, lanes 2 and 4). Conversely, the knockdown of CHCHD4 with shRNA caused a relative increase in the nuclear level of p53 (Fig. 2*A*, lanes 6 and 8). In these experiments, it is important to note that the total cellular content of p53 was not affected by either overexpression or knockdown of CHCHD4 (Fig. 2*A*, *Right*).

We next sought to image this shift in the nuclear level of p53 using confocal immunofluorescent microscopy. The overexpression of CHCHD4 caused exclusion of p53 from the nucleus after H_2O_2 treatment (Fig. 2*B*), whereas CHCHD4 depletion using shRNA increased p53 localization in the nucleus (Fig. 2*C*). Although the total cellular level of p53 protein was not affected by CHCHD4 (Fig. 2*D*), the subcellular partitioning of p53 affected its nuclear activity, as confirmed by measurement of the mRNA and protein levels of *p21*, the prototypical p53 target gene. In a p53-dependent manner, CHCHD4 expression decreased the H_2O_2-stimulated expression of *p21*, whereas CHCHD4 knockdown increased p21 levels relative to their controls (Fig. 2*D*). Also consistent with CHCHD4 regulation of p53 nuclear localization and function, overexpression of CHCHD4, which prevents p53 accumulation in the nucleus, decreased nuclear DNA integrity, whereas CHCHD4 depletion increased it (Fig. S2).

CHCHD4 Regulates p53 Function in the Mitochondria. Because multiple studies have shown that p53 participates in maintaining mtDNA, we investigated whether CHCHD4-mediated p53 importation might affect mtDNA repair as well. To test this idea, we used a PCR-based assay (quantitative long PCR) that has proven to be sensitive in detecting mtDNA breaks (25). In the presence of stable CHCHD4 overexpression or knockdown by lentiviral transduction, the cells were exposed to 200 μM H_2O_2 for 1 h to introduce mtDNA breaks, and then switched to normal tissue culture medium for the indicated times (Fig. 2 *E* and *F*). Compared with empty vector control, the overexpression of CHCHD4 significantly increased the recovery of mtDNA integrity at 6 h after H_2O_2 treatment in $p53^{+/+}$ cells, whereas no significant recovery was seen in $p53^{-/-}$ cells even up to 24 h (Fig. 2*E*). Conversely, CHCHD4 depletion decreased mtDNA integrity at both 0 h and up to 24 h after H_2O_2 treatment (Fig. 2*F*). Modulation of CHCHD4 levels caused a similar pattern of mtDNA integrity recovery after oxidative damage in primary human myoblasts, validating the results in the HCT116 cell line (Fig. S1 *B* and *C*). These findings demonstrate the importance of the disulfide relay system in maintaining mtDNA, as was also observed in a mitochondrial myopathy syndrome involving mutated CHCHD4 oxidase gene *GFER* (26). In addition, CHCHD4 promoted mitochondrial respiration and cell growth as reported previously (Fig. S3) (27). Consistent with the interaction between CHCHD4 and p53, the increase in oxidative metabolism associated with CHCHD4 overexpression was dependent on the presence of p53 (Fig. S3*A*).

Mutated p53 Can Translocate to Mitochondria and Retain mtDNA Repair Activity. We also wondered whether mutated forms of p53 can translocate into the mitochondria and mediate the repair of damaged mtDNA. We examined two well-characterized mutant forms of p53: the "hotspot" germline mutant p53 R175H

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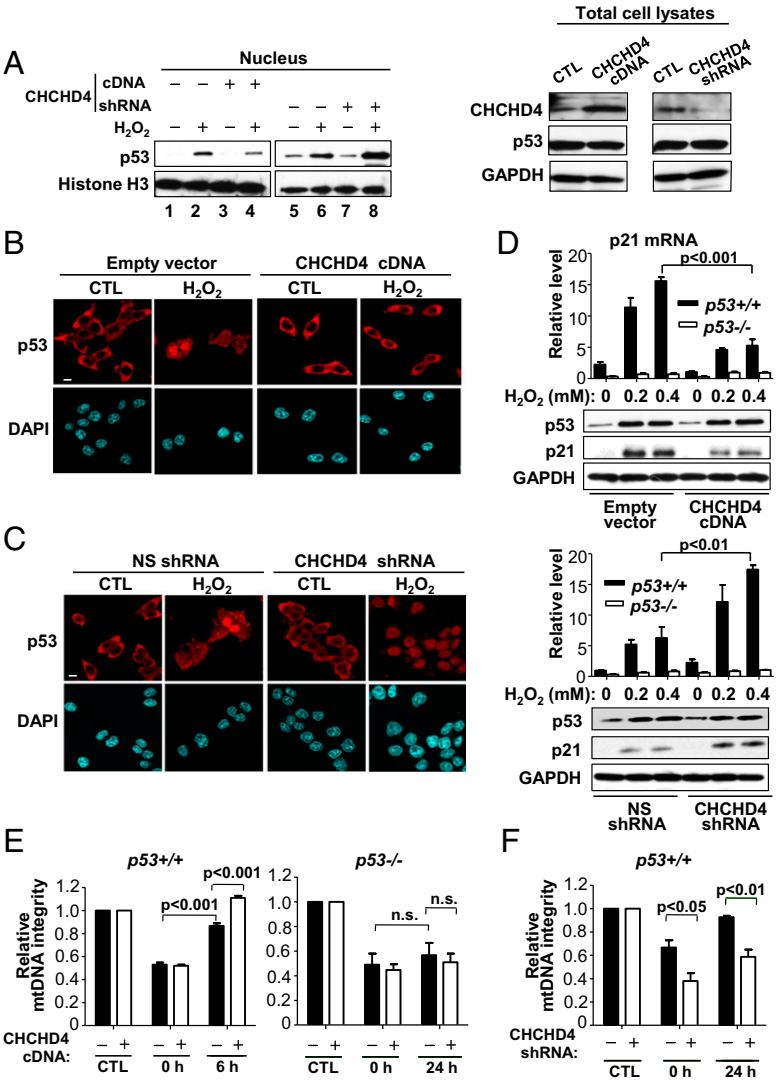

Fig. 2. CHCHD4 expression modulates the subcellular localization and activity of p53 in response to oxidative stress. (*A*) Nuclear p53 Western blot of WT HCT116 cells transduced with CHCHD4 cDNA or shRNA lentivirus after exposure to oxidative stress (200 μM H_2O_2 for 1 h). Total cell lysates (*Right*) show relative CHCHD4 levels compared with control (CTL). (*B*) Confocal immunofluorescent localization of p53 (red) in CHCHD4-overexpressing cells after oxidative stress versus no treatment (CTL). DAPI stain (blue) shows nuclei. (*C*) p53 (red) localization in WT cells with knockdown of CHCHD4 versus nonspecific (NS) shRNA. (*D*) p21 mRNA levels in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells transduced with CHCHD4 cDNA (*Upper*) or shRNA (*Lower*) lentivirus and treated with H_2O_2 for 1 h. p21 and p53 protein levels in the corresponding samples of $p53^{+/+}$ cells are shown as well. (*E*) $p53^{+/+}$ (*Left*) and $p53^{-/-}$ (*Right*) cells transduced with empty vector (–) or CHCHD4 cDNA lentivirus, treated with 200 μM H_2O_2 for 1 h, washed, and allowed to recover in normal medium for the indicated times. mtDNA integrity of treated cells relative to nontreated cells (CTL) is shown. (*F*) mtDNA integrity of WT cells transduced with nonspecific or CHCHD4 shRNA lentivirus, treated with H_2O_2, and allowed to recover for 24 h. Values are mean ± SE. ns, nonsignificant. (Scale bars: 10 μm.)

and the somatic mutant p53 C135Y. p53 R175H retained its ability to bind CHCHD4 like WT p53, whereas this interaction was abolished in p53 C135Y (Fig. 3*A*, lanes 4, 8, and 12). It is notable that the C135Y mutation of p53 results in the loss of one of its two intramolecular disulfide bonds typically present in substrates of CHCHD4 (19). Despite similar overexpression levels of both p53 mutants in the nucleus and cytoplasm, confocal immunofluorescent imaging confirmed the lack of p53 C135Y protein colocalization with CHCHD4 in the mitochondria, in marked contrast to p53 R175H, which displayed merged fluorescent signals consistent with CHCHD4 interaction (Fig. 3*B*, merge). Furthermore, depletion of CHCHD4 prevented p53 R175H localization in the mitochondria and resulted in an immunofluorescent pattern similar to that seen in mutant p53 C135Y protein (Fig. S4, merge images).

We next examined the mtDNA repair capacity of the mutant forms of p53. In contrast to p53 C135Y-expressing cells, $p53^{-/-}$ cells transduced with p53 R175H lentivirus gained the capacity to repair mtDNA after H_2O_2 treatment, which was further augmented by CHCHD4 overexpression (Fig. 3*C*). To extend the results of these in vitro experiments, we compared the mtDNA integrity of tissues from $p53^{+/+}$, $p53^{-/-}$, and homozygous *p53 R172H* (*p53H/H*, homologous to the human p53 R175H mutation) mice. The skeletal muscle, liver, and heart tissues of $p53^{-/-}$ mice showed a pattern of lower mtDNA integrity compared with $p53^{+/+}$ and *p53H/H* mice, indicating that the p53 R172H mutant retains mtDNA repair activity (Fig. 3*D*).

In Vivo Effects of CHCHD4-Mediated Translocation of p53 into Mitochondria. Although our in vitro results show that the subcellular redistribution of p53 by modulation of CHCHD4

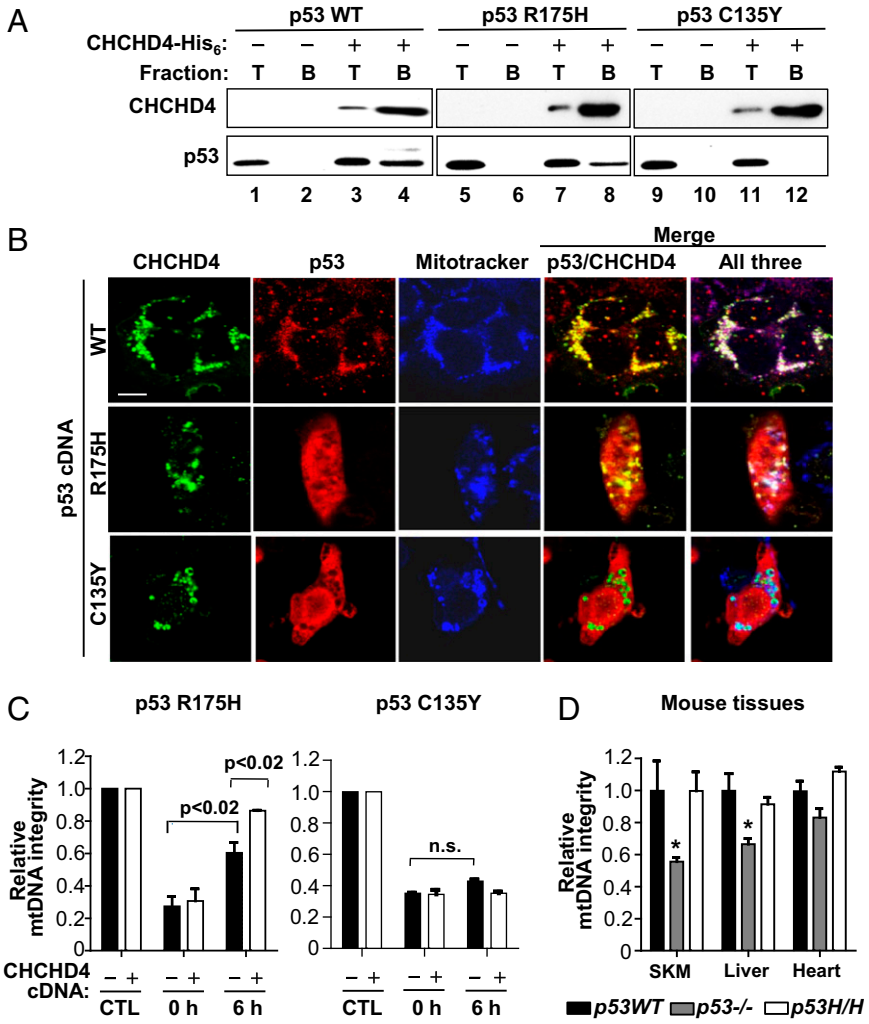

Fig. 3. Effect of p53 mutation on its CHCHD4 interaction, mitochondrial localization, and activity in mtDNA repair. (*A*) WT, R175H, or C135Y p53 cDNA was transiently cotransfected with CHCHD4-His_6 or empty vector plasmid in $p53^{-/-}$ HCT116 cells. CHCHD4-His_6 was isolated by passing the cell lysate over a Ni-NTA column. Total lysate (T) and column-bound (B) fractions were evaluated by Western blot analysis. (*B*) Confocal immunofluorescent imaging of CHCHD4 (green), p53 (red), and mitochondria (MitoTracker, blue). (*C*) $p53^{-/-}$ cells were transduced with p53 R175H or p53 C135Y lentivirus and then transfected with empty vector or CHCHD4 cDNA. The cells were treated with 200 μM H_2O_2 for 1 h, washed, and allowed to recover in normal medium for 6 h. mtDNA integrity of treated cells relative to nontreated cells (CTL) is shown. (*D*) Relative mtDNA integrity in skeletal muscle (SKM), liver, and heart of $p53^{-/-}$ and homozygous *p53 R172H* (*p53H/H*) mice compared with $p53^{+/+}$ mice. Values are mean ± SE. n.s., nonsignificant. $^*P < 0.05$. (Scale bar: 10 μm.)

expression can affect nuclear p53 activity, we wished to investigate whether such a mechanism may be functional in vivo. To do this, we used adenovirus to deliver control GFP or CHCHD4 cDNA to the liver of mice via tail vein injection. Successful transduction was confirmed by protein expression (Fig. 4*A*). Subsequent doxorubicin treatment of both groups of mice resulted in similar increases in the levels of DNA damage marker γ-H2AX (Fig. 4*A*), but the p53-dependent increases in p21 mRNA and protein levels were markedly attenuated by CHCHD4 overexpression (Fig. 4 *A* and *B*). In contrast, increased CHCHD4 expression in the liver not only increased the basal level of mtDNA integrity in a p53-dependent manner, but also significantly protected mtDNA from damage by doxorubicin treatment (Fig. 4*C*). In the $p53^{-/-}$ mice, the low basal level of mtDNA integrity in liver was not further decreased by this doxorubicin treatment regimen (Fig. 4*C*). The spleen tissue of these mice, which cannot be transduced by i.v. injection of adenovirus, served as a negative control to demonstrate the specific in vivo effect of CHCHD4 on p21 expression (Fig. S5).

Discussion

In the present study, we have delineated a specific molecular mechanism by which p53 can translocate into the mitochondria using a disulfide relay system that is conserved in plants and animals (15). The interaction of p53 with this vectorial import system is dependent on respiration and can be activated by oxidative stress, consistent with the notion that p53 evolved to provide basic adaptive functions against environmental stresses and is involved in redox homeostasis (20, 28). This finding is unique among other proposed p53 translocation mechanisms, in that it is dependent on oxidative metabolism, a function that p53 also has been shown to promote in vivo (29, 30). Furthermore, our identification of a defined molecular mechanism by which p53 can translocate into mitochondria highlights the direct role of p53 in preserving the integrity of the mitochondrial genome, which is more susceptible to oxidative damage owing to its structure and proximity to electron transfer reactions.

The subcellular partitioning of p53 into the mitochondria, with the potential to substantially affect its nuclear activity, has important implications for basic investigations of the function of this widely studied protein. For example, p53 binding to specific genomic DNA sequences has been shown to be disrupted under conditions of severe oxidative stress that involves disulfide bond formation between C135 and C141 (18). Given that CHCHD4 substrates typically contain two nonconsecutive intramolecular disulfide bonds (19), along with our observation that the p53 C135Y mutation abrogates p53 translocation, it is tempting to speculate that this type of molecular mechanism could seamlessly integrate the redistribution of nuclear DNA-bound p53 to the mitochondria in response to oxidative stress.

Our findings also may provide insight into cancer biology. A recent study elegantly showed that CHCHD4 promotes mitochondrial function and tumor growth, largely attributable to hypoxic HIF1α stabilization, and that its increased expression

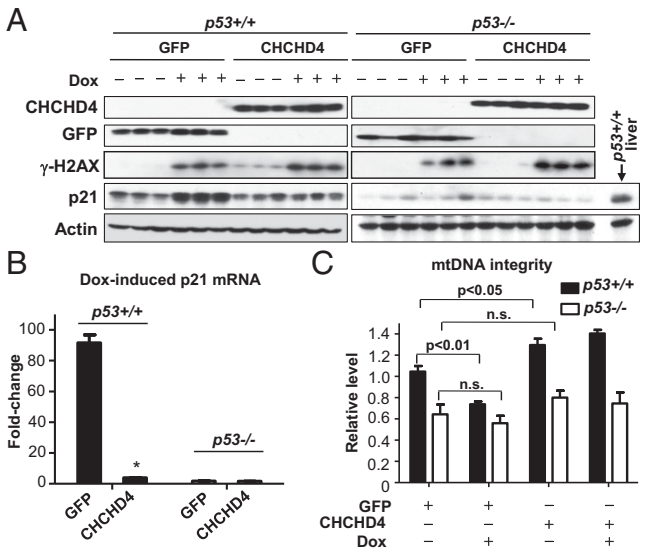

Fig. 4. Effect of CHCHD4 overexpression on *p21* expression and mtDNA integrity in liver. (*A*) At 4 d after tail vein injection of CHCHD4 or control GFP adenovirus to transduce the liver, $p53^{+/+}$ and $p53^{-/-}$ mice were treated with doxorubicin (Dox) and killed after 18 h. CHCHD4, γ-H2AX, and p21 protein levels in liver were subjected to Western blot analysis to determine CHCHD4 transduction by adenovirus, DNA damage, and p53 activation in the nucleus by doxorubicin treatment, respectively. Three mice in each group are shown. A $p53^{+/+}$ liver sample was included with the $p53^{-/-}$ samples as a positive control. (*B*) p21 mRNA level in liver tissue was measured as a marker of nuclear p53 transcriptional activity. (*C*) Relative mtDNA integrity in the liver tissues was measured as a marker of mitochondrial p53 activity. Values are mean ± SE. $*P < 0.001$.

correlates with poor prognosis in cancer patients (27). We have also found that CHCHD4 promotes cancer cell growth, but that the improvement in mitochondrial metabolism is dependent on p53 in the HCT116 cell line (Fig. S3 *A* and *C*). We recently reported that germline mutations of *TP53* in humans can increase oxidative metabolism in skeletal muscle, which may be attributed in part to interactions of CHCHD4 with mutated p53, such as the R175H mutation (30). In addition, our study raises the possibility that CHCHD4 also directly impacts p53-regulated pathways important for cancer cell proliferation, such as down-regulation of the cell cycle inhibitor p21. Further exploration of how the partitioning of p53 activity between the nucleus and the mitochondria by CHCHD4 affects cell metabolism, proliferation, or death is likely to provide more insight into p53 regulation in cancer and aging.

Materials and Methods

Cell Culture. The WT HCT116 cell line was obtained from American Type Culture Collection and cultured in McCoy's 5A medium with 10% FBS. The $p53^{-/-}$ HCT116 cell line was a generous gift from Bert Vogelstein, Johns Hopkins University, Baltimore, MD. Generation and characterization of the $SCO2^{-/-}$ HCT116 cell line have been described previously (23, 24). Primary skeletal muscle myoblasts expressing WT p53 were obtained from human subjects after approval by the National Institutes of Health internal review board and after participants provided written informed consent (NCT00406445). Myoblasts were isolated and cultured as described previously (30).

Mice. All mice were maintained and handled in accordance with the National Heart, Lung, and Blood Institute's Animal Care and Use Committee protocol. The WT, $p53^{-/-}$ (Jackson Laboratories), and *p53 R172H* [strain 01XL9; National Cancer Institute's Frederick Mouse Repository (31)] mice were of the C57BL/6 strain or backcrossed at least five generations into C57BL/6 background.

Adenovirus Generation and Injection into Mice. The adenovirus-expressing mouse CHCHD4 was prepared using the AdEasy Adenoviral Vector system (Agilent Technologies) following the manufacturer's protocol. The mice were infected with 5×10^7 pfu of adenovirus per gram of body weight. Adenovirus-expressing GFP served as negative control (Ad-GFP; Vector Biolabs).

Gene Knockdown and Overexpression. In this paper, CHCHD4 refers to the CHCHD4.1 isoform that is homologous to yeast MIA40 and is endogenously expressed by the HCT116 cells (27). The following plasmids were used: nonspecific or human CHCHD4 shRNA for gene knockdown (Open Biosystems), pReceiver-Lv105-human CHCHD4 cDNA (GeneCopoeia) for overexpression, and pReceiver-M77-human CHCHD4-His_6 cDNA for His-tag pull-down experiments. Lentiviruses for overexpression and knockdown were prepared using the respective plasmids (Sigma-Aldrich) following the manufacturer's protocol. Cells were incubated with virus (multiplicity of infection ~1) for 24 h, followed by selection with 2 μg/mL puromycin. CHCHD4-His_6 plasmid was transfected into HCT116 cells using Fugene HD transfection reagent (Roche). The QuikChange II Kit (Stratagene) was used to introduce point mutations into the CPC motif (substitute C53 or/and C55 with serine residues) of pReceiver-M77-human CHCHD4-His_6 plasmid. The primer sequences used for site-directed mutagenesis and shRNA are provided below.

Antibodies and Western Blot Analysis. The following antibodies were used: human p53 in Western blot analysis and immunofluorescent imaging (monoclonal antibody DO-1, sc-126; Santa Cruz Biotechnology), human CHCHD4 in Western blot analysis and immunofluorescent imaging (polyclonal antibody H107, sc-98628; Santa Cruz Biotechnology), actin (Santa Cruz Biotechnology), mouse CHCHD4 (ab87033 Abcam), mouse p21 (OP76; Calbiochem), human p21 (OP64; Calbiochem), GADPH (Ambion), and VDAC (Rockland). Proteins were resolved and subjected to Western blot analysis using standard SDS/PAGE and ECL (GE Healthcare).

Immunofluorescent Imaging. Cells were allowed to attach to glass-bottomed tissue culture ware (Lab-Tek) for 24 h, after which they were transfected with the indicated constructs for 48 h and then stained with MitoTracker Deep Red FM (Invitrogen). This was followed by fixation in 4% paraformaldehyde PBS (Electron Microscopy Sciences) and permeabilization in 0.1% Triton X-100 PBS. Immunofluorescent labeling was performed by blocking with 5% serum (of secondary antibody species) at room temperature, followed by incubation with p53 or CHCHD4 primary antibody at 4 °C overnight and with Alexa Fluro 488- or Alexa Fluro 555-labeled secondary antibody (Invitrogen) for at 37 °C for 1 h. PBS washes were performed between all steps. Images were captured with a confocal laser scanning microscope (Olympus Fluoview FV10i).

His_6-Tag Pull-Down Assay. HCT116 cells transfected with CHCHD4-His_6 or mutated CHCHD4-His_6 were lysed in buffer A (1% Triton X-100, 300 mM NaCl, 1 mM PMSF, and 50 mM sodium phosphate; pH 8.0) supplemented with 10 mM imidazole, and then centrifuged at 13,000 × *g* for 20 min. The supernatant was loaded to a Ni-NTA spin column (Qiagen) for isolating the his_6-tagged CHCHD4 and its associated proteins according to the manufacturer's instructions. The column was sequentially washed with buffer A containing 20 mM, 40 mM, and 80 mM imidazole, then eluted with buffer A containing 150 mM imidazole, which was designated as the binding fraction. Proteins in the total lysate and binding fraction were resolved by SDS/PAGE under reducing conditions (10 mM DTT) and subjected to Western blot analysis with the indicated antibodies.

Real-Time RT-PCR. Real-time RT-PCR was performed with an ABI HT7900 thermal cycler (Applied Biosystems). The results were normalized by measuring average cycle threshold (Ct) ratios between *p21* and the housekeeping gene *EIF3F (TIF)* (32). PCR primer sequences are provided below.

mtDNA and Nuclear DNA Integrity Assay Using Quantitative Long PCR. To cause DNA damage, cells were treated with 200 μM H_2O_2 in serum-free medium at 37 °C for 1 h, or 10 wk-old tumor-free male mice were injected via the tail vein with doxorubicin (20 mg/kg body weight). Total DNA was extracted from cells or mouse liver using the DNeasy Blood and Tissue Kit (Qiagen). For the mtDNA assay, long (8.9 kb in human, 10 kb in mouse) and short mtDNA fragments reflecting mtDNA integrity and copy number, respectively, were amplified using the GeneAmp XL PCR kit as described previously (25). For the nuclear DNA assay, a 13.5-kb human nuclear DNA fragment was amplified using the primer pair listed below. The PCR products were quantified by PicoGreen (Molecular Probes) fluorescence, and relative mtDNA integrity was calculated as a ratio of the amounts of long vs. short PCR fragments. Primers sequences are provided below.

Metabolic Studies. Whole-cell oxygen consumption and extracellular acidification were measured as markers of oxidative phosphorylation and glycolysis, respectively, using a Seahorse Bioscience XF24 metabolic analyzer (23).

Primer Sequences.

Human CHCHD4 shRNA:

5′-TGGTTACAAATATGATTCG-3′

Human CHCHD4 site-directed mutagenesis:

CHCHD4 C53S (SPC mutant)

Forward: 5′-CATTAACTGGAACAGCCCATGCCTTG-3′

Reverse: 5′-CAAGGCATGGGCTGTTCCAGTTAATG-3′

CHCHD4 C55S (CPS mutant)

Forward: 5′-CATTAACTGGAACTGCCCAAGCCTTGGGGGAATGGCCAG-3′

Reverse: 5′-CTGGCCATTCCCCCAAGGCTTGGGCAGTTCCAGTTAATG-3′

CHCHD4 C53S and C55S (SPS mutant)

Forward: 5′-GAAACATTAACTGGAACAGCCCAAGCCTTGGGGGAATGGC-3′

Reverse: 5′-GCCATTCCCCCAAGGCTTGGGCTGTTCCAGTTAATGTTTC-3′

p21 mRNA quantification:

Mouse p21

Forward: 5′-AGGGCAACTTCGTCTGGGAG-3′

Reverse: 5′-TTGGAGACTGGGAGAGGGCA-3′

Human p21

Forward: 5′-CCCGTCTCAGTGTTGAGCCTT-3′

Reverse: 5′-GTTCCGCTGCTAATCAAAGTGC-3′

mtDNA integrity assay:

Mouse mtDNA

10-kb long fragment

Forward: 5′-GCCAGCCTGACCCATAGCCATATTAT-3′

Reverse: 5′-GAGAGATTTTATGGGTGTATTGCGG-3′

Short fragment

Forward: 5′-CCCAGCTACTACCATCATTCAAGT-3′

Reverse: 5′-GATGGTTTGGGAGATTGGTTGATG-3′

Human mtDNA

8.9-kb long fragment

Forward: 5′-TCTAAGCCTCCTTATTCGAGCCGA-3′

Reverse: 5′-TTTCATCATGCGGAGATGTTGGATGG-3′

Short fragment

Forward: 5′-CCCCACAAACCCCATTACTAAACCCA-3′

Reverse: 5′-TTTCATCATGCGGAGATGTTGGATGG-3′

Human nuclear DNA integrity assay:

13.5-kb long fragment

Forward: 5′-CGAGTAAGAGACCATTGTGGCAG-3′

Reverse: 5′-GCACTGGCTTAGGAGTTGGACT-3′

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