Coiled Coil Domain-containing Protein 56 (CCDC56) Is a Novel Mitochondrial Protein Essential for Cytochrome c Oxidase Function*

Susana Peralta1, Paula Clemente1, Álvaro Sánchez-Martínez2, Manuel Calleja3, Rosana Hernández-Sierra3, Yuichi Matsushima3, Cristina Adán4, Cristina Ugalde3, Miguel Ángel Fernández-Moreno4, Laurie S. Kaguni3, and Rafael Garese1,4

From the Departamento de Bioquímica, Instituto de Investigaciones Biomédicas “Alberto Sols” Universidad Autónoma de Madrid (UAM)-Consejo Superior de Investigaciones Científicas (CSIC), Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER) Facultad de Medicina, 28029 Madrid, Spain, 1Instituto de Investigación Sanitaria 12 de Octubre (I+12), 28041 Madrid, Spain, 3Centro de Biología Molecular “Severo Ochoa,” CSIC-UAM, 28049 Madrid, Spain, and 4Department of Biochemistry and Molecular Biology and Center for Mitochondrial Science and Medicine, Michigan State University, East Lansing, Michigan 48824-1319

Background: Cytochrome c oxidase (COX), the final enzyme of the mitochondrial electron transport chain, requires several assembly factors for its proper function.

Results: ccdc56 knock-out flies showed developmental delay, lethality, and a dramatic decrease in the levels/activity of COX.

Conclusion: CCDC56 protein is necessary for COX function and for viability in flies.

Significance: Drosophila CCDC56 is a novel putative COX assembly factor conserved in humans.

In Drosophila melanogaster, the mitochondrial transcription factor B1 (d-<i>mtTFB1</i>) transcript contains in its 5′-untranslated region a conserved upstream open reading frame denoted as CG42630 in FlyBase. We demonstrate that CG42630 encodes a novel protein, the coiled coil domain-containing protein 56 (CCDC56), conserved in metazoans. We show that <i>Drosophila</i> CCDC56 protein localizes to mitochondria and contains 87 amino acids in flies and 106 in humans with the two proteins sharing 42% amino acid identity. We show by rapid amplification of cDNA ends and Northern blotting that <i>Drosophila</i> CCDC56 protein and <i>mtTFB1</i> are encoded on a <i>bona fide</i> bicistronic transcript. We report the generation and characterization of two <i>ccdc56</i> knock-out lines in <i>Drosophila</i> carrying the <i>ccdc56<sup>Δ<sub>N</sub></sup></i> and <i>ccdc56<sup>Δ<sub>111</sub></sup></i> alleles. Lack of the CCDC56 protein in flies induces a developmental delay and 100% lethality by arrest of two larval development at the third instar. <i>ccdc56</i> knock-out larvae show a significant decrease in the level of fully assembled cytochrome <i>c</i> oxidase (COX) and in its activity, suggesting a defect in complex assembly; the activity of the other oxidative phosphorylation complexes remained either unaffected or increased in the <i>ccdc56</i> knock-out larvae. The lethal phenotype and the decrease in COX were partially rescued by reintroduction of a wild-type <i>UAS-ccdc56</i> transgene. These results indicate an important role for CCDC56 in the oxidative phosphorylation system and in particular in COX function required for proper development in <i>D. melanogaster</i>. We propose CCDC56 as a candidate factor required for COX biogenesis/assembly.

Cytochrome <i>c</i> oxidase (COX)<sup>5</sup> or complex IV (EC 1.9.3.1) is the terminal enzyme of the electron transport chain, and it catalyzes electron transfer from reduced cytochrome <i>c</i> to molecular oxygen. Most cellular ATP is produced in mitochondria by the oxidative phosphorylation (OXPHOS) system comprising the electron transport chain complexes (plus two electron carriers, coenzyme Q, and cytochrome <i>c</i>) and the multitimeric ATP synthase (complex V) (1). The energy released from the oxidation of carbohydrates and lipids is converted to reducing power (NADH + H<sup>+</sup> and FADH<sub>2</sub>) in the mitochondrial matrix. The electron transport chain couples electron transfer from NADH and FADH<sub>2</sub> to molecular oxygen with proton translocation from the matrix to the mitochondrial intermembrane space by complexes I, III, and IV. This proton translocation generates an electrochemical gradient that is used by complex V to generate ATP from ADP and inorganic phosphate.

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2 cia Lain Entralgo Grant PI 08/0021 (to C. U.).
3 A postdoctoral fellow from CIBERER. Present address: Dept. of Neurology, University of Miami Miller School of Medicine, Miami, FL 33136.
4 Present address: Dept. of Biomedical Sciences, Medical Research Council Centre for Developmental and Biomedical Genetics, University of Sheffield, Sheffield S10 2TN, UK.
5 Present address: Dept. of Mental Retardation and Birth Defect Research, National Inst. of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan.
6 To whom correspondence should be addressed: Inst. de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC, c/ Arturo Duperier 4, 28029 Madrid, Spain. Tel.: 34-91-4975452; Fax: 34-91-5854401; E-mail: rafael.garese@uam.es.
7 The abbreviations used are: COX, cytochrome c oxidase; CCDC56, coiled coil domain-containing protein 56; RACE, rapid amplification of cDNA ends; OXPHOS, oxidative phosphorylation; mt, mitochondrial; <i>mtTFB1</i>, mitochondrial transcription factor B1; uORF, upstream open reading frame; AEL, after egg laying; <i>d</i>-<i>CCDC56</i>, Drosophila CCDC56; TES, 2-[2-hydroxyethyl]-1-bis[hydroxyethyl]ethyl]amino[ethanesulfonic acid; Bis-Tris, 2-[bis[2-hydroxyethyl]amino]-2-(hydroxymethyl)propane-1,3-diol; Tb, Tubbly; qRT-PCR, quantitative RT-PCR.

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Eukaryotic COX is a heteromeric enzyme of dual genetic origin (2,3). The catalytic core of the enzyme is composed of three subunits encoded in the mitochondrial DNA (mtDNA): mt-CO1, mt-CO2, and mt-CO3. The structural subunits that surround the catalytic core are encoded by the nuclear genome (4). The nuclear genome-encoded subunits must be imported into the mitochondria, processed, and assembled together with the mtDNA-encoded subunits to form the holoenzyme. The nuclear genome-encoded subunits are necessary for the assembly/stability of the holoenzyme (5) and to regulate the catalytic activity of complex IV (6,7).

More than 20 assembly factors required for correct COX function have been described in yeast; albeit the specific function of many of these factors remains elusive (for a review, see Ref. 8). Assembly factors are proteins involved in the biogenesis of the complex that are not present in the mature complex. They are involved in different biological processes, for example in the biogenesis and/or insertion of prosthetic groups (9–12), regulation of mt-CO1 translation (13), and stabilization of the mt-CO1 and mt-CO3 transcripts (14,15). Translation of genes in bacteria and Archaea occurs in polycistronic messenger RNA, whereas in Eukaryota, the majority of genes are transcribed monocistronically (16). However, there are some exceptions in Eukaryota where genes are transcribed in polycistronic messages, and in general, these polycistronic genes tend to be involved in the same biological process as occurs in bacteria (17–20).

Mitochondrial gene expression is regulated by several nuclear-encoded proteins, including the mitochondrial transcription factor B1 (mtTFB1) (21). mtTFB1 is dual function protein that can activate mtDNA transcription in vitro (22) and act as an RNA methyltransferase in vivo (23,24). Previous work from our group in cultured Drosophila cells indicated a major role for mtTFB1 in mitochondrial translation (25). And more recently, Larsson and co-workers (26) have corroborated these data in mitochondria-mediated by mtTFB1 is required for assembly of the mitochondrial ribosome and therefore for mitochondrial translation. The mtTFB1 gene in Drosophila melanogaster was annotated as the protein-coding gene number CG7319 in the fly genome database (FlyBase). More recently, the FlyBase genome annotators have published changes affecting the annotation of the mtTFB1 gene that indicates the existence of an upstream open reading frame (uORF) in its 5′-untranslated region. The putative protein coding gene is annotated as CG42630 in the FlyBase database. Here we show that CG42630 is transcribed in a bicistronic RNA messenger with the mtTFB1 gene and is expressed in flies. BLAST analysis of the novel uORF indicated 42% amino acid identity with the human annotated coiled coil domain-containing protein 56 (CCDC56; NCBI accession number NP_001035521.1). Thus, we propose Drosophila CG42630 as the homolog of human CCDC56. Although the function of CCDC56 is unknown, it is highly conserved in higher eukaryotes. To study the function of the CCDC56 protein, we generated a D. melanogaster knock-out model by inducing genomic deletions by imprecise P element excision. Our results indicate that the CCDC56 homolog is a mitochondrial protein required for COX activity and assembly in D. melanogaster, suggesting a role as a COX assembly factor.

**EXPERIMENTAL PROCEDURES**

*Drosophila Strains and Genetics—*All fly crosses and stocks were grown at 25 °C on a standard *Drosophila* medium. ccdc56Δ/Δ and ccdc56Δ/Δ mutants were generated by inducing the transposition of the SUPor-P[kg07792] P element insertion using standard procedures (27). Deletion break points of alleles were determined by PCR followed by sequencing using specific primers (see Fig. 3, B and C). Sequences were assembled and analyzed using the Vector NTI software (Invitrogen). Transgenic lines for the plUASP-ccdc56, plUASP-mtTFB1, and plUASP-cDNA constructs were generated by the injection of Drosophila embryos (BestGene).

**Identification and Sequence Analysis of Bicistronic ccdc56-mtTFB1 cDNA and CCDC56—*CDNAS from Drosophila control larvae (w1118 and Oregon R-C) and Schneider cells were prepared using the First Choice RLM-RACE cDNA amplification kit (Ambion). 5′-Rapid amplification of cDNAs ends (RACE) was performed using the following specific primers for Drosophila mtTFB1 cDNA (CG42631; formerly CG7319): R1, R2, and R3 (depicted in Fig. 1A). RACE products were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced. Sequence analysis was performed using Vector NTI Advance 10 software (Invitrogen). Human CCDC56 (NCBI accession number NP_001035521.1) and CCDC56 homologs were identified by BLAST using the deduced amino acid sequence of the D. melanogaster CG2630 coding gene (28). Multiple sequence alignments of the predicted CCDC56 polypeptides were performed using the ClustalW 2.0.12 algorithm (29).

**Northern Blotting—*Five micrograms of total RNA from control flies were resolved on a 1.2% agarose gel and transferred to a Zeta-Probe GT membrane (Bio–Rad) following standard procedures. Invitrogen’s 0.5–10-kb RNA ladder was used as a molecular size marker. A PCR fragment of 280 bp containing the complete ccdc56 ORF (261 bp) was used as a ccdc56-specific probe. This probe was amplified by PCR from the pUASP-ccdc56 construct using primers 9558F and 9559R (see below). The specific probe for the mtTFB1 coding sequence (322 bp) was obtained by PCR amplification using the primers F9 (5′-AGACACATCCCCGACACCTCA-3′) and R4 (5′-TTTATGG-GAATTAAGTTGACGG-3′). Probes were radiolabeled with [32P]dCTP using the Amersham Bioscience Rediprime II Random Prime Labeling System (GE Healthcare) following the manufacturer’s instructions.

**Phenotyping Analysis—**We carried out 2-h egg laying from the control w1118 stock and the stable mutant stocks ccdc56Δ/Δ/TM6b-Tb and ccdc56Δ/Δ/TM6b-Tb. To determine whether mutant larvae were developmentally arrested, developmentally delayed, or merely slow growing, their mouth hooks were examined daily under the microscope from the 5th day after egg laying (AEL). Fly vials were also photographed daily.

**Bacterial Expression of Drosophila CCDC56 (d-CCDC56) and Generation of Anti-d-CCDC56 Antibody—**To express d-CCDC56 in Escherichia coli, a PCR fragment encoding the d-CCDC56 open reading frame was cloned into the pRSET-B vector (Invitrogen) cut with Ncol and HindIII. The following
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primers were used: Fw, 5′-TTCCATGGCGCGTCCGGAGCAGGGACC-3′; and Rv, 5′-AGAAGGCTCTGAAGGAGACCTTTCTGGGCTC-3′ (bold nucleotides mean restriction enzyme sites). Polyclonal antibody was generated using standard procedures.

Constructs for the Generation of Transgenic Flies—The bicistronic ccd56-mtTFB1 cDNA (1574 bp) was obtained from the cDNA clone LD40326 (GenBank™ access number AY069635) and cloned into the BglII/XbaI restriction sites in the pUASP vector. To generate the pUASP-ccd56 construct, a fragment containing exclusively the complete ccd56 ORF (261 bp) was amplified by PCR using the primers 9558F (5′-TTTAGACGCGTTTATAATGTCG-3′) and 9559R (5′-TAGGGATAACTAACC CGGACA-3′), subcloned into the pCAP vector (Roche Applied Science), and subcloned into the NotI/XbaI restriction sites in the pUASP vector. To generate the pUASP-mtTFB1 construct, mtTFB1 ORF (990 bp) was obtained by digestion with KpnI/NotI of the pBluescript II KS(−) vector (Roche Applied Science), and cloned into the NotI/XbaI restriction sites in the pUASP vector.

Drosophila CCDC56-FLAG Construct—D. melanogaster CCDC56 ORF was amplified by PCR from the pUASP-ccd56 construct indicated above using the primers F (5′-TTGGTACCATGTGGCGGTCCGGACGAGGACC-3′) and R (5′-TTGGCGCCGCCCTACATTGTCGTCACTATCCGTTTGTAGTGGG AAGACACCCTTCTGGGCTC-3′) containing the FLAG epitope at the C terminus and the KpnI/NotI sites needed for cloning into the pcDNA3 vector (Stratagene). Preparations were visualized under a Leica TCS SP2 laser-scanning microscope.

Mitochondrial Enzyme Assays and Mitochondrial RNA (mtRNA) and mtDNA Quantification—For enzymatic activity measurements, mitochondrial-enriched homogenates were prepared from ~30 third instar larval ground in SETH buffer (250 mm sucrose, 2 mm EDTA, 100 units/liter heparin, 10 mm Tris-HCl, pH 7.4), fractionated by differential centrifugation, and sonicated (6 s at 4 °C). The activities of the respiratory chain complexes I, II, III, and IV and the mitochondrial mass marker citrate synthase were measured by spectrophotometric methods as described previously (30) and expressed in nanomoles of substrate catalyzed/minute/milligram of protein. For mtRNA quantification, RNA was extracted using TRIzol reagent (Invitrogen), and 1 μg from each genotype was converted into cDNA and amplified in a 7900 Fast Real Time PCR System (Applied Biosystems) using the TaqMan probes. For relative quantification of mtDNA, genomic DNA was isolated from third instar larvae and quantified using standard methods. Ten nanograms of each DNA were used as template. TaqMan probes for mt-ND5 and mt-CO1 were used.

Immunoblotting and Subcellular Fractionation—Thirty micrograms of each mitochondrial protein extract, obtained by differential centrifugation, were separated on 10 or 15% SDS-polyacrylamide gels and transferred to Immobilon P PVDF membranes (Millipore). Filters were preincubated for 1 h in 5% skim milk in TBS, 0.1% Tween 20 followed by an overnight incubation with the corresponding primary antibody. Monoclonal antibody against porin (1:2000; voltage-dependent anion channel) was obtained from Molecular Probes, polyclonal antibody against d-CCDC56 was generated as described above (1:50), and polyclonal antibody anti-mtTFB1 was previously generated by Dr. L. S. Kaguni (1:1000) (25). For subcellular fractionation, D. melanogaster embryos were homogenized in 250 mm sucrose, 10 mm TES, 1 mm EDTA, pH 7.4 with five strokes at 1000 rpm using a motor-driven Teflon pestle. Homogenates were then centrifuged at 900 × g for 10 min at 4 °C, and the supernatant was centrifuged again at 9000 × g for 10 min at 4 °C to obtain the mitochondrial fraction (pellet) and postmitochondrial supernatant. Fifty micrograms of each fraction were loaded onto 12% SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with anti-porin (1:1000), anti-GAPDH (1:1000; Stressgene), and anti-d-CCDC56 (1:50) antibodies.

Blue Native Gel Analyses—Mitochondrial pellets isolated from larvae were resuspended in 1.5 M aminocaproic acid, 75 mm Bis-Tris, pH 7.0. Respiratory chain complexes were extracted with 2% lauryl maltoside for 20 min on ice and then centrifuged for 30 min at 4 °C. Blue native polyacrylamide gel electrophoresis (PAGE) and two-dimensional electrophoresis (two-dimensional SDS-PAGE) were performed as described (31), loading 40 μg of mitochondrial protein per lane. A 4–18% native acrylamide gradient gel was used for the first dimension, and 10% acrylamide gels were used for electrophoresis in the second dimension. Proteins were transferred overnight to a nitrocellulose membrane. Anti-mt-CO3 (Invitrogen) was used at a 1:200 dilution; polyclonal antibody against β-ATPase, generated in our laboratory (32), was used at 1:1000; and secondary
The protein CCDC56, encoded in a bicistronic transcript together with mt-TFB1 in D. melanogaster, is conserved in metazoans. A, genomic map of CG42630/ccdc56 and mtTFB1. Exons are indicated by boxes, coding regions are colored black for the CCDC56 and mtTFB1 proteins, and untranslated regions are represented in white. B, bicistronic transcript determined by 5′-RACE from control flies (w1118 and Oregon-R-C) and cultured Schneider cell cDNAs. The transcription start point identified is depicted as +1. The primers R1, R2, and R3 used are represented in A. C, bicistronic ccd56-mtTFB1 mRNA detected by Northern blot using 5 μg of RNA from w1118 (C1) and Oregon-R-C (C2) control larvae. The signal detected using a specific ccd56 probe has the same migration as the signal detected when using a probe specific for mtTFB1. D, ClustalW alignment of Drosophila CCDC56 protein with CCDC56 sequences from other metazoan species. Accession numbers are as follows: fly (D. melanogaster), FlyBase annotation CG42630-PA; zebrafish (Danio rerio), UniProtKB accession ABK877; western clawed frog (Xenopus tropicalis), UniProtKB accession Q3T0E3; human (Homo sapiens), NCBI accession number NP_001035521.1. Identical residues in all sequences (*), conserved substitutions (.), and semiconserved substitutions (.) are noted in the alignment. E, schematic diagram of the sequences of the human and D. melanogaster CCDC56 proteins showing the putative transmembrane and protein-protein interaction coiled coil domains. Hu, human; dm, D. melanogaster; aa, amino acids.

RESULTS

The D. melanogaster Gene CG42630 Is Expressed in a Bicistronic mRNA with mtTFB1 and Encodes a Novel Conserved Protein, CCDC56—A search in the Drosophila genome database (FlyBase) indicated the existence of a uORF in the 5′-UTR of the mtTFB1 transcript located in the first exon of the mRNA (Fig. 1A). This putative polypeptide is denoted as CG42630 from FlyBase, and the biological processes in which it is potentially involved are unknown. To confirm the existence of a bona fide bicistronic transcript of CG42630-mtTFB1, we isolated mRNA from Drosophila strains and from Drosophila SL2 Schneider cells and carried out RACE (Fig. 1B) using the strategy described under “Experimental Procedures.” We sequenced the cDNA clones obtained by 5′-RACE from different Drosophila strains (w1118, n = 8; Oregon R-C, n = 6) and from cultured Schneider cells (n = 6), and in all cases, sequence analyses detected a single transcript with a heterogeneous transcription start point located upstream from the CG42630 ORF (Fig. 1B). We excluded the presence of transcripts coding only for CG42630 by 3′-RACE (data not shown). These results suggest strongly that d-mtTFB1 and the putative gene, CG42630, are transcribed in the same mRNA and therefore are encoded in a bicistron. We tested the existence of this CG42630-mtTFB1 bicistronic mRNA by Northern blot in total RNA extracted from D. melanogaster flies (Fig. 1C) using two different probes: a 280-nucleotide probe specific for the CG42630 coding sequence and a 320-nucleotide probe specific for the mtTFB1 coding sequence (see “Experimental Procedures”). We detected with both probes a single signal of about the expected size of 1574 bp (Fig. 1A), confirming the existence of the CG42630-mtTFB1 bicistronic structure in D. melanogaster.

The putative CG42630 gene encodes a predicted polypeptide of 87 amino acids (FlyBase). The deduced amino acid sequence from Drosophila CG42630 was used to perform a BLAST analysis of the human genome (28), and a single protein of unknown function was identified: CCDC56. We found a 42% amino acid
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In contrast to the bicistronic structure detected in flies, human CCDC56 is encoded in a single transcript, and the gene consists of two putative domains: a single pass transmembrane domain (from amino acids 49 to 69 in *D. melanogaster*) and a protein-protein interaction coiled coil domain (from amino acids 79 to 104 in humans and from amino acids 70 to 87 in *D. melanogaster*) (Fig. 1D). We refer to the *Drosophila* CG42630 gene as *ccdc56*.

To map precisely the deletion break points, we PCR-amplified the intron in the *ccdc56* coding sequence and the first 26 nucleotides of the *mtTFB1* coding sequence without affecting the *mtTFB1* coding sequence, *ccdc56D6* and *ccdc56D11*, were selected (Fig. 3, A–C). A *Drosophila* line harboring an allele in which the *P* element had been removed, two lines carrying deletions that map specifically in the *ccdc56* coding sequence without affecting the *mtTFB1* coding sequence, *ccdc56D6* and *ccdc56D11*, were selected (Fig. 3, A–C). These lines were obtained by the same process as those harboring the deletions. Homozygous *ccdc56D6D6* and *ccdc56D11D11* flies are not viable and die in the third larval instar stage, suggesting that *ccdc56* is essential for development.

**Generation of *ccdc56* Knock-out Alleles**—To study the *in vivo* function of *CCDC56*, we generated transgenic flies harboring *ccdc56* loss-of-function alleles. To generate deletions that specifically affect the *ccdc56* gene, we mobilized the *P(SUPor-P)* element in the proximal 5′-region of the gene using standard procedures (27). From ~100 independent lines in which the *P* element had been removed, two lines carrying deletions that map specifically in the *ccdc56* coding sequence without affecting the *mtTFB1* coding sequence, *ccdc56D6* and *ccdc56D11*, were selected (Fig. 3, A–C). A *Drosophila* line harboring an allele in which the *P* element was excised precisely without removing additional DNA sequence was used as a control (designated *Control*; Fig. 3, D and E). The control flies were obtained by the same process as those harboring the deletions. Homozygous *ccdc56D6D6* and *ccdc56D11D11* flies are not viable and die in the third larval instar stage, suggesting that *ccdc56* is essential for development. *ccdc56D6D11* trans-heterozygotes showed the same lethal phenotype as homozygous *ccdc56D6D6* and *ccdc56D11D11* flies, indicating that these alleles did not complement each other, which is expected if the molecular lesion affects the same gene. To map precisely the deletion break points, we PCR-amplified and sequenced the genomic region flanking the *P* element (Fig. 3, A–C). The *ccdc56D6* allele harbors a 570-bp-long deletion that includes the ATG initiation codon and the first 23 nucleotides of the ORF of *CCDC56* (Fig. 3B). The *ccdc56D11* allele shows a larger deletion of 1168 bp comprising the complete *ccdc56* coding sequence and the first 26 nucleotides of the intron in the *ccdc56-mtTFB1* mRNA (Fig. 3C).

**Phenotype of *ccdc56D6D6* and *ccdc56D11D11***—*Drosophila* Fly Lines—Heterozygous *ccdc56D6D6*, *TM6B-Tb* and *ccdc56D11D11*, *TM6B-Tb* flies are viable and fertile and can be distinguished easily by the genetic marker *Tubby* (*Tb*; generating small pupa size) that allows classifying the progeny. When raised at 25 °C, heterozygous mutant larvae, like control larvae, reached the third larval stage at 4–5 days AEL. However, *ccdc56D6D6* and *ccdc56D11D11* homozygotes took between 10 and 12 days to reach the third larval stage, monitored by analyzing the mouth hook morphology (Fig. 3B), indicating a developmental delay. Homozygous *ccdc56D6D6* and *ccdc56D11D11* third instar larvae were much smaller than controls, remained in this stage for over 20 days, and then died before pupariation (Fig. 4A). We used the *ccdc56D11D11* mutant to perform immunocytochemical analysis in wing imaginal discs, the proliferating larval epithelial cells that form the adult wings of the fly. Mutant imaginal discs were smaller than controls. Indeed, mutant wing discs...
FIGURE 3. Molecular characterization of the ccdc56D6 and ccdc56D11 alleles. A, genomic map of the ccdc56 and mtTFB1 genes showing the P element insertion (SUPor-P[67792]; triangle). Exons are indicated by boxes, coding regions are colored black for the CCDC56 and mtTFB1 proteins, and untranslated regions are represented in white. 5'-RACE from control (w1118) and mutant (ccdc56D6 and ccdc56D11) homozygous third instar larvae cDNAs identified the transcription start points, which are depicted as +1. The break points of the deletions generated in this work for the alleles ccdc56D6 and ccdc56D11 are shown in B and C, respectively. The ratios of the mRNA as determined by 5'-RACE in the clones analyzed are represented (ccdc56D6/D6, n = 8; ccdc56D11/D11, n = 10). PCR products amplified with F5 and R4 primers (shown in A) from genomic DNA of third instar larvae are shown in D. Lane 1, DNA from the stock containing the P element SUPor-P[kg07792] as a negative control; lane 2, DNA from w1118 control flies; lane 3, DNA from excised flies without any deletion used as an additional control; lane 4, DNA from excised flies of the ccdc56D6/D6 strain showing a 570-bp deletion; lane 5, DNA from excised flies of the ccdc56D11/D11 strain showing a 1168-bp deletion. E, transcript levels determined by qRT-PCR in third instar larvae of control and deleted homozygous lines after normalizing to w1118 flies using 18S rRNA as an internal control. The two different TaqMan probes used are depicted in B. Control 1, w1118 flies; Control 2, excised flies without any deletion. Values are mean ± S.E. F, CCDC56 and mtTFB1 protein levels determined by immunoblotting of mitochondrial extracts (30 μg) of control and deleted homozygous larvae. Anti-voltage-dependent anion channel/pore antibody was used as a loading control.

FIGURE 4. Lack of CCDC56 causes arrest at the third larval stage. A, size comparison of control (w1118) and homozygous mutant third instar larvae. Homozygous larvae for both alleles were smaller than control larvae in all cases tested. B, mouth hook morphology of control third instar larvae and mutant larvae 15 days AEL indicating third instar. C, wing imaginal discs from control (w1118) and homozygous ccdc56D11 third instar larvae were dissected and immunostained with anti-phosphohistone 3 (PH-3) antibody. Mutant wing discs showed lower cell proliferation levels as compared with controls. D, anti-caspase 3 activated antibody was used to detect apoptotic signals in wing imaginal discs. Increased levels of apoptosis were observed in homozygous ccdc56D11 flies as compared with control flies. Bar size, 50 μm.
showed a decreased number of mitotic cells as seen by a reduction in the number of cells that express phosphorylated histone 3, a mitotic cell marker (Fig. 4C). ccdc56\textsuperscript{D11/D11} mutants also showed increased levels of apoptosis in the wing discs as detected with an antibody that recognizes activated caspase 3, whereas cell death in control wing discs was minimal during most of the larval development (Fig. 4D). These results indicate that two of the processes that contribute to tissue growth, cell division and cell survival, are compromised in the ccdc56\textsuperscript{D11/D11} mutant.

The Phenotype of ccdc56\textsuperscript{D6/D6} and ccdc56\textsuperscript{D11/D11} Drosophila Lines Is Produced by the Absence of CCDC56—Because both deletions remove a large 5′-region upstream of the ccdc56/mtTFB1 genes that potentially contain critical promoter elements involved in regulating their transcription, we used RT-PCR to identify truncated transcripts encoding the mtTFB1 gene in the mutant lines. Interestingly, in both the homozygous D6 and D11 lines, the levels of truncated ccdc56/mtTFB1 transcript ranged between 20 and 30% as compared with the wild-type controls (Fig. 3E). Detailed analysis using 5′-RACE revealed the presence of a family of transcripts originating from the 5′-upstream region close to the break points (Fig. 3, B and C), indicating clearly that these regions still contain promoter activity. All the detected transcripts expressed in the mutant lines contained the complete d-mtTFB1 coding sequence (Fig. 3, B and C), but as expected, they lacked the complete ccdc56 coding sequence. Accordingly, no CCDC56 was detected by immunoblot analysis of mitochondrial extracts from ccdc56\textsuperscript{D6/D6} and ccdc56\textsuperscript{D11/D11} larvae, indicating that both are ccdc56-null alleles (Fig. 3F); in contrast, mtTFB1 is present in mitochondrial extracts prepared from the mutant larvae (Fig. 3F).

To demonstrate further that the lethal phenotype observed in the mutants isolated in our screen was due exclusively to the loss of CCDC56 function, we overexpressed independently ccdc56 or mtTFB1 in both D6 and D11 homozygous genetic backgrounds. We cloned the ccdc56 and mtTFB1 coding sequences in the pUASP vector and generated several UAS-ccdc56 and UAS-mtTFB1 transgenic lines (see “Experimental Procedures”). We selected two independent lines for each transgene that were viable upon homozygosis, and we generated stable stocks carrying these UAS constructs in homozygous genetic backgrounds. The UAS-ccdc56 and UAS-mtTFB1 lines expressed their respective genes at a level similar to the corresponding transgenic lines carrying the UAS-ccdc56 and UAS-mtTFB1 constructs (Fig. 3). To test if the expression of the transgenic UAS constructs reduced the lethal phenotype of the ccdc56\textsuperscript{D6/D6} and ccdc56\textsuperscript{D11/D11} mutants, we crossed these lines to the ccdc56\textsuperscript{D6/D6} and ccdc56\textsuperscript{D11/D11} mutant strains. We found that CCDC56 expression partially rescues the mutant lethality phenotype. A, flies homozygous for the ccdc56\textsuperscript{D11} allele reach only the pupal stage when they carry the UAS-ccdc56 and the arm-GAL4 transgenes on chromosome II. Larvae and pupae homozygous for the allele ccdc56\textsuperscript{D11} are of normal size. Larvae and pupae heterozygous for the deletion (genotype ccdc56\textsuperscript{D11}/TM6B-Tb) carrying one copy of the Tb marker are smaller. B, qRT-PCR of CCDC56, bicistron, and mtTFB1 mRNA relative to 18S rRNA from homozygous ccdc56\textsuperscript{D11} third instar larvae combined with the different UAS transgenes and with or without the ubiquitous arm-GAL4 driver. The three TaqMan probes used are depicted in the scheme. Data represent the mean ± S.E. of at least three independent determinations (*, p < 0.05; **, p < 0.01; Student’s t test). C, immunoblot of mitochondrial extracts (30 μg) from homozygous ccdc56\textsuperscript{D11} third instar larvae of the genotypes indicated incubated with polyclonal anti-mtTFB1 and anti-CCDC56 antibodies and with monoclonal anti-voltage-dependent anion channel/porin antibody.
TABLE 1

Rescue analysis of ccdc56<sup>D11</sup>/ccdc56<sup>D11</sup> flies

| Genotype, chromosome II | No. of ccdc56<sup>D11</sup>/TM6B-Tb pupae scored | No. of ccdc56D11/ccdc56<sup>D11</sup> pupae scored | Total no. of pupae scored | Mean (%)* ccdc56<sup>D11</sup>/ccdc56<sup>D11</sup> pupae scored/expected ± S.E. |
|-------------------------|-----------------------------------------------|-----------------------------------------------|-------------------------|-------------------------------------------------|
| +/+                     | 1502                                          | 1306                                          | 1502                    | 0                                               |
| +/arm-GAL4              | 1527                                          | 1306                                          | 1527                    | 0                                               |
| UAS-ccdc56-1/+          | 1622                                          | 1622                                          | 1622                    | 0                                               |
| UAS-ccdc56-1/arm-GAL4   | 1823                                          | 1786                                          | 1823                    | 0                                               |
| UAS-ccdc56-2/+          | 1786                                          | 1786                                          | 1786                    | 0                                               |
| UAS-mtTFB1-1/+          | 2039                                          | 2039                                          | 2039                    | 0                                               |
| UAS-mtTFB1-1/arm-GAL4   | 1984                                          | 1984                                          | 1984                    | 0                                               |
| UAS-mtTFB1-2/+          | 1629                                          | 1629                                          | 1629                    | 0                                               |
| UAS-mtTFB1-2/arm-GAL4   | 1626                                          | 1626                                          | 1626                    | 0                                               |

* Homozygous ccdc56<sup>D11</sup>/ccdc56<sup>D11</sup> pupae scored/expected × 100 of at least three replicate experiments. Homozygous ccdc56<sup>D11</sup>/ccdc56<sup>D11</sup> pupae expected are 1/3 of the total progeny of the cross. The Tb marker enables the progeny classes to be distinguished.

rescue lethality at the third larval stage for mutants carrying the D6 or D11 deletion allele (to simplify, only data are shown regarding the ccdc56<sup>D11</sup> allele; Table 1 and Fig. 5A). The presence in a mutant background of a copy only of the arm-GAL4 driver or a copy only of the UAS-ccdc56 construct did not rescue mutant lethality. Accordingly, all pupae scored in the vials were heterozygous for the mutant allele (Table 1 and Fig. 5A).

Quantification of transcript levels by qRT-PCR in homozygous third instar larvae of the different genotypes showed that the arm-GAL4 driver restores ccdc56 mRNA levels to 73.9 ± 0.1% of controls in a D11 mutant background (Fig. 5B, white bars). Despite this increase in mRNA levels, CCDC56 protein measured by immunoblot analysis was increased only to 15% of control levels (Fig. 5C). This result indicates that the induction of low levels of CCDC56 is sufficient to rescue the developmental arrest of the ccdc56<sup>D11/D11</sup> mutant, permitting pupation of the larvae and most of the metamorphosis program of the flies (Fig. 5A). The fact that the rescue of lethality was not complete may be explained by the slight increase of the CCDC56 protein obtained under these conditions. Accordingly, by restoring CCDC56 to control levels (by inducing the expression of the bicistron), we obtained a stronger rescue of lethality: 96.77 ± 1.6% of the ccdc56<sup>D11/D11</sup> mutant progeny reached the pupal stage, and 34.5 ± 4.5% reached the adult stage. As expected, the transcript levels assessed with the probe that recognizes the bicistronic mRNA were nearly absent in the D11 homozygous larvae for all transgene combinations as the D11 deletion (shown in Fig. 3C) includes part of the recognition sequence for this probe (Fig. 5B, gray bars, and Fig. 3E, Probe 1).

ccdc56 Knock-out Flies Show a Severe Isolated Cytochrome c Oxidase Deficiency—To study the effect of the absence of CCDC56 on mitochondrial function, we measured the OXPHOS enzyme activities in control and homozygous mutant third instar larvae. Both ccdc56<sup>D11/D11</sup> and ccdc56<sup>D6/D6</sup> mutant larvae showed a dramatic reduction in COX (complex IV) activity (Fig. 6A). The decrease in COX activity was almost complete when activities were normalized with respect to citrate synthase activity, an indicator of total mitochondrial mass (data not shown). This decrease in COX activity in ccdc56<sup>D11/D11</sup> mutant larvae was confirmed by blue native PAGE (Fig. 7A). Interestingly, the enzyme activities of the remaining OXPHOS complexes were either unaffected or increased significantly (complexes I and II in ccdc56<sup>D11/D11</sup> mutants; Fig. 6A), a result that may reflect a compensatory response to the dramatic reduction of COX activity in the mutants. Most interestingly, a huge increase (4–5-fold) in mtDNA levels was also observed in mutant mitochondria as compared with controls (Fig. 6B). In addition, a moderate increase was observed in the steady-state levels of mtRNA transcripts (Fig. 6C), including the small ribosomal RNA (rRNA-12 S) and the cytochrome c oxidase transcripts mt-CO1, mt-CO2, and mt-CO3 that are increased 2–2.5-fold (Fig. 6C). The increased mtRNA transcript levels in both mutants were confirmed by Northern blot analyses (data not shown).

The ubiquitous expression of the UAS-ccdc56 construct in a ccdc56<sup>D11/D11</sup> genetic background induced a significant increase in COX enzyme activity (37.4 ± 0.043 versus 19.5 ± 0.040%; Fig. 7C). As expected, expression of the UAS-mtTFB1 construct under the same conditions had no effect, showing levels of COX activity similar to those of the mutants (14.3 ± 0.043%; Fig. 7C). These results suggest strongly that CCDC56 is required for cytochrome c oxidase function in D. melanogaster.

ccdc56 Knock-out Flies Show a Severe Reduction of Fully Assembled Complex IV—Because the only OXPHOS complex affected in ccdc56 knock-out flies is complex IV, we next explored by blue native PAGE analysis whether the assembly of this complex was affected in the mutants. Several antibodies against mammalian mitochondrial and nuclearly encoded COX subunits were tested against Drosophila protein extracts, but unfortunately, most showed little or no cross-reactivity (data not shown). To determine the levels of fully assembled complex IV in our mutant flies, we performed two-dimensional blue native PAGE followed by immunoblot analysis with an anti-mt-CO3 antibody that was able to recognize Drosophila mt-CO3. In ccdc56<sup>D11/D11</sup> and ccdc56<sup>D6/D6</sup> mitochondria, we observed a dramatic decrease in the levels of fully assembled holo-COX (indicated by complex S4; Fig. 7A). However, we did not detect the accumulation of any subcomplex or putative assembly intermediate in our mutants at least under the conditions tested (Fig. 7A). To demonstrate that the absence of CCDC56 function was responsible for the mutant complex IV assembly defect, we attempted to rescue this phenotype by inducing the ubiquitous expression of a UAS-ccdc56 transgene in a mutant background. Consistent with our hypothesis, we observed a recovery of fully assembled complex IV levels in mutants expressing UAS-ccdc56 (Fig. 7A, third panel) but not when we overexpressed mitochondrial translation factor B1 (Fig. 7A, fourth panel). This result indicates that CCDC56 is required for the proper assembly and/or stability of mitochondrial complex IV in D. melanogaster.
**DISCUSSION**

We have identified in *D. melanogaster* a novel mitochondrial protein, CCDC56, which is evolutionarily well conserved in metazoans. CCDC56 belongs to the coiled coil domain-containing family of proteins, and although its function is unknown, we found that loss of CCDC56 results in a severe isolated enzyme deficiency and assembly defect of mitochondrial cytochrome *c* oxidase. This indicates that CCDC56 plays a critical role in the biogenesis and activity of complex IV and is therefore essential for the function of the OXPHOS system.

In *D. melanogaster*, CCDC56 is annotated in a single transcription unit together with mitochondrial transcription factor B1 (FlyBase). BLAST analyses showed that this organization is also present in the other 11 *Drosophila* species for which sequence data are available (data not shown). Northern blot and RACE experiments demonstrated that *ccd56* and *mtTFB1* are encoded on the same functional bicistronic transcript in *D. melanogaster*. Although the presence of operon-like structures is not frequent in eukaryotes, a comparative evolutionary study of 12 *Drosophila* genomes predicted the presence of 123 novel polycistronic transcripts (34). More recently, another comparative genomics approach using *D. melanogaster* and *Anopheles gambiae* transcript annotations and dipteran expressed sequence tags was performed to identify transcripts with uORFs. Interestingly, in dipterans, conserved uORFs occur preferentially in transcripts encoding mitochondrial proteins and methyltransferases (35). The uORF usually encodes proteins smaller than those encoded in the main ORF. Some examples in *Drosophila* are the translocase of the inner membrane 10, Tim 10 (FlyBase annotation CG9878), and the translocase of the inner membrane 9b, Tim 9b (*CG17767*). It is possible that at least some of these uORFs are vestiges of ancient prokaryotic operons that originated in the mitochondrion and were transferred to the nuclear genome over time. Another possible explanation would be that these structural organizations favor a coordinated regulation of genes involved in similar biochemical pathways (16).

We further investigated whether this novel peptide is targeted to the mitochondrial compartment, like mtTFB1, by transfecting HeLa cells with a recombinant *d*-CCDC56 FLAG-tagged protein. We observed a clear mitochondrial localization of CCDC56, and in addition, we detected by immunoblotting the endogenous CCDC56 only in the mitochondrial fraction. Therefore and in agreement with the results obtained in the comparative genomics study, we describe here a new case of a bicistronic transcript in which both proteins have a mitochondrial function that is demonstrated to be functional in *vivo*.

We explored the consequences of the lack of function of mitochondrial protein CCDC56 by generating two independent *Drosophila* knock-out lines by inducing the excision of a P element located in the promoter region of the *ccd56-mtTFB1*
more, we have demonstrated that both mutant lines retained mtTFB1-encoding transcripts and mtTFB1 protein although in lower levels as compared with controls. Finally, we used the UAS-GAL4 system (36) to promote independent expression of CCDC56 or mtTFB1 in a mutant background. Overexpression of CCDC56 but not mtTFB1 rescued all mutant phenotypes: developmental delay, larval lethality, decreased complex IV enzyme activity, and reduction in the levels of fully assembled complex IV. Previous results have shown that mtTFB1 is essential for mitochondrial translation and more specifically for the stability of the small subunit of the mitochondrial ribosomes (12 S rRNA) (25, 26). Thus, if the functionality of mtTFB1 were affected in these alleles, we would expect a broader mitochondrial phenotype, not simply an isolated complex IV deficiency. Biochemical measurements showed no decrease in the other respiratory chain complex activities in mutant animals or in complex V levels detected by blue native PAGE, which were even higher in the mutants. Taken together, our results indicate clearly that the phenotypes exhibited by the mutants were due exclusively to loss of CCDC56 function.

The main biochemical phenotype of the lack of CCDC56 is a severe isolated complex IV deficiency, suggesting a role for CCDC56 in complex IV activity and consequently in the proper function of the OXPHOS system. Interestingly, in ccdc56 knock-out animals, the OXPHOS defect elicits a compensatory response that causes a significant increase in mtDNA, in mitochondrial transcript levels, in OXPHOS activities in complexes I and II, and in levels of fully assembled complex V. The molecular nature of this retrograde signaling is presently unknown.

The OXPHOS defect caused by the complex IV deficiency may be responsible for the developmental delay and 100% lethality in the third larval instar observed in ccdc56 knock-out individuals. Mutant wing discs showed a reduced size, a decrease in cell proliferation, and an increase in apoptosis levels as compared with controls. Larval to adult metamorphosis, which occurs during the pupal phase, is a high-energy-requiring process. Thus, defects in the OXPHOS system and consequently defects in ATP production may be critical for successful completion of this developmental phase. This is supported by the phenotype observed in several other Drosophila mitochondrial gene mutants. For example, knockdown of mtTFB2, which encodes the mitochondrial transcription factor B2, or mutations in mitochondrial single-stranded DNA-binding protein, both involved in mtDNA replication, also cause a developmental delay and a developmental arrest in the third larval instar (37, 38).

Complex IV assembly was studied initially by Nijtmans et al. (39), and subsequently by others (40) using blue native gel electrophoresis in human cell lines or tissues from patients with genetic defects in assembly factors (for reviews, see Refs. 41 and 42). These studies describe complex IV biogenesis as a sequential process involving four intermediates, S1 to S4. The process starts with the incorporation of the prosthetic groups into mt-CO1 to form the subassembly intermediate S1. The last intermediate, S4, forms a holo-COX protein, both involved in mtDNA replication, also cause a developmental delay and a developmental arrest in the third larval instar (37, 38).


genes. Its imprecise excision removed part of the flanking DNA and therefore generated specific deletions. Transcripts detected by RACE in the ccdc56D16 knock-out line lack the first 23 nucleotides and therefore the translation start codon of ccdc56, whereas no ccdc56-containing transcripts were detected in the ccdc56D11 knock-out line. As expected, CCDC56 was not detected in mitochondrial extracts from any of the mutant lines, indicating a total absence of CCDC56 protein in homozygous ccdc56D16 and ccdc56D11 animals. Further-

FIGURE 7. Loss of ccdc56 induces a complex IV assembly defect. A, two-dimensional blue native (BN)/SDS-PAGE analysis of mitochondrial extracts immunoblotted with an antibody against the mitochondrial encoded subunit mt-CO3 of the cytochrome c oxidase complex. Mitochondrion-enriched extracts were prepared from third instar larvae of the following genotypes: 1, control w1118; 2, mutant ccdc56D11/D11; 3, mutant ubiquitously expressing the UAS-ccdc56 transgene (arm-GAL4/UAS-ccdc56; ccdc56D11/D11); 4, mutant larvae expressing the UAS-mtTFB1 transgene under the same condition (arm-GAL4/UAS-mtTFB1; ccdc56D11/D11). The lack of fully assembled holo-COX exhibited by the ccdc56D11/D11 mutant is rescued partially by overexpression of the UAS-ccdc56 transgene. S4 is the fully assembled complex IV. The previously identified mt-CO3-containing COX subcomplex S3 is indicated. B, first dimension of duplicate blue native PAGE of mitochondrial extracts incubated with a polyclonal antibody against complex V (C-V) as a loading control for the two-dimensional blue native/SDS-PAGE shown in A. C, complex IV enzyme activity measured in mitochondrial extracts from homozygous ccdc56D11/D11, third instar larvae carrying the indicated constructs on chromosome II. Data were normalized to control larvae (w1118) and represent the mean S.E. (n = 3; *, p < 0.05; Student’s t test).
by two-dimensional blue native electrophoresis using an anti-mt-CO3 antibody. As expected, fully assembled complex IV levels were restored partially by the ubiquitous expression of a UAS-ccdc56 transgene in the mutant background. These results indicate that CCDC56 is essential for the formation or stabilization of complex IV. No subassembly intermediates were detected under these conditions. mt-CO3 is incorporated into the S3 intermediate as indicated by the absence of fully assembled holo-COX and the accumulation of S1 and S2 in cultured fibroblasts from patients lacking mt-CO3 (Ref. 43, and for reviews, see Refs. 42 and 44). Unfortunately, we tried several antibodies against other complex IV subunits with no success, so we cannot rule out the possible accumulation of other subassembly intermediates not containing mt-CO3 and thus a role for CCDC56 in the early steps of complex IV biogenesis.

Although the function of CCDC56 in flies remains unknown, because ccdc56 knock-out strains have extremely low levels of fully assembled complex IV, it is tempting to suggest that CCDC56 constitutes a new complex IV assembly factor. In this regard, CCDC56 might participate in various steps of complex IV biogenesis: synthesis of mitochondrial COX subunits, synthesis of COX cofactors, the stability of different COX subunits, or their assembly into a functional holoenzyme. We suggest that it may function as a translational activator similar to TACO1 (13) or as a membrane insertion factor like OXA1 (45). Future experiments are warranted to explore these possibilities. Notably, an increasing number of essential assembly factors for the biogenesis of a functional cytochrome c oxidase have been identified in Saccharomyces cerevisiae (4). We found by BLAST analysis that CCDC56 homologs are present in metazoans that show high conservation levels. However, no CCDC56 homolog was detected in yeast. The absence of a CCDC56 homolog in yeast might be due to low evolutionary conservation or could suggest the presence of novel levels of regulation of the complex IV assembly process in metazoans, thus highlighting the importance of using different model systems for the identification of the complete mitochondrial proteome.

In conclusion, we have identified CCDC56 as a novel mitochondrial protein essential for cytochrome c oxidase activity in D. melanogaster and hence OXPHOS function. The high degree of conservation between the human and Drosophila proteins suggests strongly that the biological function of CCDC56 has been preserved in metazoans, making CCDC56 a new candidate gene to study in human mitochondrial diseases involving isolated cytochrome c oxidase deficiency.

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