

Coiled-Coil Domain Containing protein 56 (CCDC56) is a novel mitochondrial protein essential for cytochrome c oxidase function\*

Susana Peralta<sup>1,3</sup>, Paula Clemente<sup>1</sup>, Álvaro Sánchez-Martínez<sup>1,4</sup>, Manuel Calleja<sup>5</sup>, Rosana Hernández-Sierra<sup>1</sup>, Yuichi Matsushima<sup>6,7</sup>, Cristina Adán<sup>1</sup>, Cristina Ugalde<sup>2</sup>, Miguel Ángel Fernández-Moreno<sup>1</sup>, Laurie S. Kaguni<sup>6</sup> and Rafael Garesse<sup>1\*</sup>.

- 1- Departamento de Bioquímica, Instituto de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC. Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER) Facultad de Medicina, Universidad Autónoma de Madrid, Spain.
- 2- Instituto de Investigación Sanitaria 12 de Octubre (i+12) Madrid, Spain.
- 3- Present address: Department of Neurology, University of Miami Miller School of Medicine, Miami, Florida 33136, USA.
- 4- Present address: Department of Biomedical Sciences, MRC Centre for Developmental and Biomedical Genetics, University of Sheffield, Sheffield S10 2TN, UK.
- 5- Centro de Biología Molecular “Severo Ochoa” CSIC-UAM, Madrid, Spain.
- 6- Department of Biochemistry and Molecular Biology, and Center for Mitochondrial Science and Medicine, Michigan State University, East Lansing, MI 48824-1319, USA.
- 7- Present address: Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan.

Running Title: Isolated COX deficiency in *Drosophila*

\* Corresponding author. Rafael Garesse, Instituto de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC. c/ Arturo Duperier 4, 28029-Madrid, Spain. Email: [rafael.garesse@uam.es](mailto:rafael.garesse@uam.es) Phone +34-914975452. Fax: +34-91-5854401

**Accession codes.** GenBank: CCDC56, NP\_001035521.1. Flybase ID: FBgn0261353, CG42630.

**Keywords:** CCDC56, cytochrome *c* oxidase, OXPHOS function, Complex IV, mitochondria, *Drosophila*

## CAPSULE

**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.

## SUMMARY

In *Drosophila melanogaster*, the mitochondrial transcription factor B1 (*mtTFB1*) transcript contains in its 5'-untranslated region a conserved upstream

**Open Reading Frame (uORF) 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 *Drosophila* 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 (RACE) and Northern-blotting that *Drosophila* CCDC56 protein and *mtTFB1* are encoded on a bona fide bicistronic transcript. We report the generation and characterization of two *ccdc56* knockout lines in *Drosophila* carrying the *ccdc56*<sup>D6</sup> and *ccdc56*<sup>D11</sup> alleles. Lack of the CCDC56 protein in flies induces a developmental delay and 100% lethality by arrest of larval development at the third instar. *ccdc56* knockout larvae show a**

**significant decrease in the level of fully-assembled cytochrome c oxidase (COX) and in its activity, suggesting a defect in complex assembly; the activity of the other Oxidative Phosphorylation (OXPHOS) complexes remained either unaffected or increased in the *ccdc56* knockout larvae. The lethal phenotype and the decrease in COX were rescued partially by reintroduction of a wild-type *UAS-CCDC56* transgene. These results indicate an important role for CCDC56 in the OXPHOS system and in particular in COX function, required for proper development in *Drosophila melanogaster*. We propose CCDC56 as a candidate factor required for COX biogenesis/ assembly.**

## INTRODUCTION

Cytochrome c oxidase (COX) or Complex IV (EC 1.3.9.1) is the terminal enzyme of the electron transport chain, and catalyzes electron transfer from reduced cytochrome c 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 c) and the multimeric 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 the 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.

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 (nDNA)(4). The nDNA-encoded subunits must be imported into the mitochondria, processed and assembled together with the mtDNA-encoded subunits to form the holoenzyme. The nDNA-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 (reviewed in 8). Assembly factors are proteins involved in the biogenesis of the complex, which 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).

Transcription 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 occur 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 rRNA 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 coworkers have corroborated this data in mammals, where they showed methylation of the 12S rRNA mediated by mtTFB1 is required for assembly of the mitochondrial ribosome, and therefore for mitochondrial translation (26). The *mtTFB1* gene in *Drosophila melanogaster* was annotated as the protein coding gene number *CG7319* in the fly genome database (<http://flybase.org>). 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 (<http://flybase.org>). Here we show that *CG42630* is transcribed in a bicistronic RNA messenger with the *mtTFB1*

gene and is expressed in flies. Blast of the novel uORF indicated 42% amino acid identity with the human annotated Coiled-Coil Domain Containing protein 56, (CCDC56; NP\_001035521.1). Thus, we propose *Drosophila* CG42630 as the homologue 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 *Drosophila melanogaster* knockout 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 *Drosophila 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. *ccdc56*<sup>D6</sup> and *ccdc56*<sup>D11</sup> mutants were generated by inducing the transposition of the SUPor-P[kg07792] P element insertion using standard procedures (27). Deletion breakpoints of alleles were determined by PCR followed by sequencing using specific primers (Fig 3B-C). Sequences were assembled and analyzed using the Vector NTI software (Invitrogen). Transgenic lines for the *pUASP-CCDC56*, *pUASP-mtTFB1* and *pUASP-cDNAbi* 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 (*w*<sup>1118</sup> and *Oregon R-C*) and Schneider cells were prepared using the *First Choice RLM-RACE* cDNA amplification kit (Ambion). 5' RACE was performed using the following specific primers for *Drosophila mtTFB1* cDNA (CG42631, formerly CG7319): R1, R2 and R3, depicted in Fig1A.. RACE products were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced. Sequence analysis was performed using Vector NTI Advance 10 (Invitrogene Software). Human CCDC56 (NP\_001035521.1) and CCDC56 homologues were identified by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) 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 µg 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'-AGCACATCCCGGACACCTCA-3' and R4: 5'-TTTAGGGGAATTAGCTTGACG-3'. Probes were radiolabeled with [<sup>32</sup>P]-dCTP using the Amersham Rediprime II Random Prime Labeling System (GE Healthcare) following the manufacturer's instructions.

### Phenotyping analysis

We carried out 2-hour egg lays from the control *w*<sup>1118</sup> stock and the stable mutant stocks *ccdc56*<sup>D6</sup>/*TM6b-Tb* and *ccdc56*<sup>D11</sup>/*TM6B-Tb*. To determine if mutant larvae were developmentally arrested, developmentally delayed, or merely slow growing, their mouth hooks were examined daily under the microscope from the fifth day after egg laying (AEL). Fly vials were also photographed daily.

### Bacterial expression of 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 *NcoI* and *HindIII*. Primers used were: Fw: 5'-TTCCATGGCGGCGTCGGAGCAGGGACC-3' and Rv: 5'-AGAAGCTTCTAGGAAGACACCTTCTTGGGCTC-3'. Polyclonal antibody was generated using standard procedures.

### Constructs for the generation of transgenic flies

The bicistronic *ccdc56-mtTFB1* cDNA (1574 bp) was obtained from the cDNA clone LD40326 (GenBank AY069635) and cloned into the *BglIII/XbaI* restriction sites of the pUAST transformation vector to generate the pUAST-*cDNA-bi* construct. To generate the pUASP-*ccdc56* vector for transformation, a fragment containing exclusively the complete *ccdc56* ORF (261 bp), was amplified by PCR using the following primers: 9558F: 5'-TTTAGCAGCGTTTATAATGTCTG-3' and 9559R: 5'-TAGGGATAACTAACGCGGACA-3', subcloned into the pCAP vector (Roche) and cloned 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 (Stratagene) containing the *mtTFB1* cDNA and cloned into the pUASP vector for transformation.

### Drosophila CCDC56-FLAG construct

*D. melanogaster* CCDC56 ORF was amplified by PCR from the pUASP-*ccdc56* construct indicated above, using the following primers: F 5'-TTGGTACCATGTCTGGCGTCGGAGCAGG GACC-3' and R 5'-TTGCGGCCGCTACTTGTCGTCATCGT CTTTGTAGTCGGAAGACACCTTCTTGGG CTCC-3' containing the FLAG epitope at the C-terminal and the *KpnI/NotI* sites needed for cloning into the mammalian expression vector pcDNA3 (Invitrogen). Fidelity of the clones was confirmed by sequencing.

### Transfection and generation of CCDC56-FLAG overexpressing cell lines

Human HeLa cells were grown in DMEM (GIBCO-BRL) supplemented with 5% fetal bovine serum (GIBCO-BRL).  $1.5 \times 10^5$  HeLa cells were plated on coverslips and transfected with 2  $\mu$ g of the pcDNA3-*d-ccdc56*-FLAG construct. Lipofectamine (Invitrogen) was used as a transfection reagent, following the manufacturer's instructions.

### Immunohistochemistry

To label the mitochondrial compartment, cells were incubated for 30 min with 250 nM of the mitochondrial dye MitoTracker red (Invitrogen)

24 h after transfection, washed, and fixed for 15 min in 2% paraformaldehyde. Primary anti-FLAG antibody (1:1000, Stratagene) and secondary Alexa Fluor 488 anti-mouse antibody (1:200, Molecular Probes) were used. Images were collected using a Confocal microscope (Leica).

Imaginal discs from third instar larvae of each genotype were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. They were blocked in PBS, 1% bovine serum albumin, 0.3% Triton X-100 for 1 h, incubated with the primary antibody overnight at 4°C (dilution 1:50), washed, and incubated with the appropriate secondary antibody for 2 h at room temperature in the dark (dilution 1:200). Finally, they were washed and mounted in Vectashield (Vector Laboratories). Primary antibodies used were rabbit anti-phosphohistone 3 (Sigma-Aldrich) and rabbit anti-caspase 3 (Cell Signaling Technology). Secondary antibodies were coupled to the fluorochromes Alexa Fluor 647 or Alexa Fluor 555 (Invitrogen). Preparations were visualized under a Leica TCS SP2 laser-scanning microscope.

### Mitochondrial enzyme assays and mtRNA and mtDNA quantification

For enzymatic activity measurements, mitochondria-enriched homogenates were prepared from approximately 30 third instar larvae ground in SETH buffer (250 mM sucrose, 2 mM EDTA, 100 U/L heparin, 10 mM Tris-HCl, pH 7.4), fractionated by differential centrifugation and sonicated (6s, 15 microns 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 previously described (30) and expressed in nanomoles of substrate catalyzed per minute per milligram of protein. For mtRNA quantification, RNA was extracted using TRIzol reagent (Invitrogen) and 1  $\mu$ g 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 ng of each DNA were used as template. Taqman probes for *mt-ND5* and *mt-COI* were used.

### Immunoblotting/ subcellular fractionation

Thirty  $\mu$ g of each mitochondrial protein extract, obtained by differential centrifugation, were separated on 10% or 15% SDS-PAGE gels and transferred to Immobilon P PVDF membranes (Millipore). Filters were pre-incubated 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, VDAC) 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, *Drosophila melanogaster* embryos were homogenized in 250 mM sucrose, 10 mM TES and 1mM EDTA pH 7.4 with 5 strokes at 1000 rpm using a motor-driven Teflon pestle. Homogenates were then centrifuged at 900g for 10 minutes at 4°C and the supernatant was centrifuged again at 9000g for 10 minutes at 4°C to obtain the mitochondrial fraction (pellet) and post-mitochondrial supernatant. Fifty  $\mu$ g of each fraction were loaded onto 12% SDS-PAGE 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% laurylmaltoside for 20 min on ice, and then centrifuged for 30 min at 4°C. Blue Native Polyacrilamide Gel Electrophoresis (BN-PAGE) and 2D electrophoresis (2D SDS-PAGE) were performed as described (31), loading 40  $\mu$ g 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  $\beta$ -ATPase, generated in our lab (32) was used at 1:1000, and secondary goat-anti mouse antibody IgG-HRP (provided by Zymed Laboratories) was used at 1:2000.

### Statistical analysis

Statistical data analysis was performed with Prism 4.03 software (GraphPad Software). One-way ANOVA (plus Bonferroni post-test) and Student's t-test were used to determine statistical significance of the results, which was assumed at  $p < 0.05$ .

## 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 (<http://flybase.org>) indicated the existence of an upstream Open Reading Frame (uORF) in the 5'-UTR of the *mtTFB1* transcript located in the first exon of the mRNA (Fig1A). This putative polypeptide is denoted as *CG42630* in 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 Rapid Amplification of cDNAs ends (RACE, Fig1B), using the strategy described in Materials and Methods. We sequenced the cDNA clones obtained by 5'-RACE from different *Drosophila* strains (*w*<sup>1118</sup>, 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* uORF (Fig1B). 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 (Fig1C) using two different probes: a 280 nucleotide (nt) probe specific for the *CG42630* coding sequence, and a 320 nt probe specific for the *mtTFB1* coding sequence (see Materials and Methods). We detected with both probes a single signal of about the expected size of 1574 bp (Fig1A), confirming the existence of the *CG42630-mtTFB1* bicistronic structure in *D. melanogaster*.

The putative *CG42630* gene encodes a predicted polypeptide of 87 amino acids (<http://flybase.org>). 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: the Coiled-Coil Domain Containing protein 56, CCDC56. We found a 42% amino acid identity between *D. melanogaster* and its human homolog. CCDC56 has two putative domains: a single-pass transmembrane domain (aa58 to aa78 in humans and aa49 to aa69 in *D. melanogaster*) and a protein-protein interaction coiled-coil domain (from aa79 to aa104 in humans and from aa70 to aa 87 in *D. melanogaster*) (Fig1E). We refer to the *Drosophila CG42631* gene as *ccdc56*.

In contrast to the bicistronic structure detected in flies, human CCDC56 is encoded in a single transcript and the gene is located on chromosome 17q.21.31 (Entrez gene ID 28958). Human CCDC56 protein contains 106 aa protein (Swiss Prot Q9Y2R0). Orthologous CCDC56 proteins are present in metazoans showing a high degree of conservation (Fig1D), but they are absent in yeast and plants. No mitochondrial signal peptide was detected in the amino acid sequences of the *Drosophila* and human CCDC56 proteins using the bioinformatic programs TargetP and iPSORT.

### CCDC56 is a mitochondrial protein.

In prokaryotes, operons encode factors that are usually involved in the same metabolic pathway or biological process (33). Because mtTFB1 is an essential protein involved in mitochondrial translation, we first examined the possibility that CCDC56 is also located in mitochondria (25,26). We cloned the *Drosophila* CCDC56 ORF tagged with the Flag epitope (FLAG) at the carboxyterminal end (*d*-CCDC56-FLAG), and transiently-transfected cultured HeLa cells (Fig2). Using anti-FLAG antibodies, we observed a clear colocalization with the mitochondrial-specific dye MitoTracker Red, indicating that the tagged CCDC56 version has a mitochondrial localization (Fig2C).

In order to detect the endogenous protein in the fly, we generated a polyclonal antibody against the *Drosophila* CCDC56 polypeptide. Immunoblot analysis of

subcellular fractions of wild type embryos demonstrated that endogenous *Drosophila* CCDC56 (*d*-CCDC56) is expressed and localized to the mitochondrial fraction (Fig2D). The protein shows an electrophoretic mobility that corresponds to a molecular mass of approximately 10 KDa, in accordance with the predicted size of the *Drosophila* CCDC56 protein. Monoclonal anti-porin antibody was used as a mitochondrial marker.

### Generation of *ccdc56* knockout alleles

To study the *in vivo* function of CCDC56 we generated transgenic flies harbouring *ccdc56* loss-of-function alleles. To generate deletions that affect specifically the *ccdc56* gene, we mobilized the *P{SUPor-P}mtTFB1<sup>[KG07792]</sup>* transposon located in the proximal 5'-region of the gene using standard procedures (27). From approximately 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, *ccdc56<sup>D6</sup>* and *ccdc56<sup>D11</sup>*, were selected (Fig3A-C). A *Drosophila* line harbouring an allele in which the P element was excised precisely without removing additional DNA sequence was used as a control (designated Control 2, Fig3D-E). The control flies were obtained by the same process as those harboring the deletions. Homozygous *ccdc56<sup>D6/D6</sup>* and *ccdc56<sup>D11/D11</sup>* flies are not viable and die in the third larval instar stage, suggesting that *ccdc56* is essential for development. *ccdc56<sup>D6/D11</sup>* trans-heterozygotes showed the same lethal phenotype as homozygous *ccdc56<sup>D6/D6</sup>* and *ccdc56<sup>D11/D11</sup>* 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 breakpoints, we PCR-amplified and sequenced the genomic region flanking the P element (Fig3A-C). The *ccdc56<sup>D6</sup>* allele harbors a 570 bp-long deletion that includes the ATG-initiation codon and the first 23 nucleotides of the ORF of CCDC56 (Fig3B). The *ccdc56<sup>D11</sup>* allele shows a larger deletion of 1068 bp, comprising the complete *ccdc56* coding sequence and the first 26 nucleotides of the intron in the *ccdc56*-*mtTFB1* mRNA (Fig 3C).

### Phenotype of *ccdc56<sup>D6/D6</sup>* and *ccdc56<sup>D11/D11</sup>* *Drosophila* fly lines

Heterozygous *ccdc56*<sup>D6</sup>/TM6B-Tb and *ccdc56*<sup>D11</sup>/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 After Egg Laying (AEL). However *ccdc56*<sup>D6/D6</sup> and *ccdc56*<sup>D11/D11</sup> homozygotes took between 10-12 days to reach the third larval stage, monitored by analyzing the mouth hook morphology (Fig4B), indicating a developmental delay. Homozygous *ccdc56*<sup>D6/D6</sup> and *ccdc56*<sup>D11/D11</sup> third instar larvae were much smaller than controls, remained in this stage for over 20 days and then died before pupariation (Fig4A). We used the *ccdc56*<sup>D11/D11</sup> 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 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 (Fig4C). *ccdc56*<sup>D11/D11</sup> mutants also showed increased levels of apoptosis in the wing discs as detected with an antibody that recognizes activated Caspase3, whereas cell death in control wing discs was minimal during most of the larval development (Fig4D). These results indicate that two of the processes that contribute to tissue growth, cell division and cell survival, are compromised in the *ccdc56*<sup>D11/D11</sup> mutant.

### **The phenotype of *ccdc56*<sup>D6/D6</sup> and *ccdc56*<sup>D11/D11</sup> *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 20-30% as compared to the wild type controls (Fig3E). Detailed analysis using 5'-RACE revealed the presence of a family of transcripts originating from the 5'-upstream region close to the breakpoints (Fig3B-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 (Fig3B-C) but as expected, they lacked the complete *ccdc56* coding sequence. Accordingly, no CCDC56 was detected by immunoblot analysis of mitochondrial extracts from *ccdc56*<sup>D6/D6</sup> and *ccdc56*<sup>D11/D11</sup> larvae, indicating that both are *ccdc56* null alleles (Fig3F); in contrast, mtTFB1 is present in mitochondrial extracts prepared from the mutant larvae (Fig3F).

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 Materials and Methods). We selected two independent lines for each transgene that were viable upon homozygosis, and we generated stable stocks carrying these UAS constructs in homozygosis on chromosome II, and the *ccdc56*<sup>D6</sup> or *ccdc56*<sup>D11</sup> alleles balanced over the TM6B-Tb chromosome on chromosome III. To direct the expression of the transgenic UAS constructs we used the ubiquitous arm-GAL4 driver, in which expression of the transcription activator factor GAL4 is driven by the *armadillo* promoter ( $\beta$ -catenin homolog in mammals). We found that homozygous mutant larvae carrying one copy each of the UAS-CCDC56 and the arm-GAL4 transgenes developed beyond the third larval stage, reaching the pupal stage (Fig5A). Two independent transgenic lines expressing CCDC56 produced  $62.8 \pm 1.8$  % and  $55.9 \pm 4.8$ % of the homozygous mutant pupae progeny expected for a neutral allele (Table 1). However, the UAS-mtTFB1 construct directed by the same driver was not able to 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 Fig5A). 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 Fig5A).

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  $73.9\% \pm 0.1$  of controls in a *D11* mutant background (Fig5B, white bar). Despite this increase in mRNA levels, CCDC56 protein measured by immunoblot analysis was increased only to 15% of control levels (Fig5C). 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 (Fig5A). 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:  $96.77\% \pm 1.6$  of the *ccdc56*<sup>D11/D11</sup> mutant progeny reached the pupal stage and  $34.5\% \pm 4.5$  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 Fig3C) includes part of the recognition sequence for this probe (Fig5B, grey bars; Fig3E probe1).

#### ***ccdc56* knockout 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 cytochrome *c* oxidase (COX, complex IV, CIV) activity (Fig6A). 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 (Fig7A). 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, Fig6A), 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 to controls (Fig6B). In addition, a moderate increase was observed in the steady-state levels of mitochondrial RNA (mtRNA) transcripts (Fig6C), including the small ribosomal RNA (rRNA-12S) and the cytochrome *c* oxidase transcripts *mt-CO1*, *mt-CO2* and *mt-CO3* that are increased 2-to-2.5-fold (Fig6C). 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 ( $37.4\% \pm 0.043$  vs  $19.5\% \pm 0.040$ ; Fig7C). As expected, expression of the *UAS-mtTFB1* construct under the same conditions had no effect, showing similar levels of COX activity as the mutants ( $14.3\% \pm 0.043$  Fig7C). These results suggest strongly that CCDC56 is required for cytochrome *c* oxidase function in *Drosophila melanogaster*.

#### ***ccdc56* knockout flies show a severe reduction of fully-assembled complex IV**

Because the only OXPHOS complex affected in *ccdc56* knockout flies is complex IV, we next explored by Blue Native-PAGE analysis if the assembly of this complex was affected in the mutants. Several antibodies against mammalian mitochondrial and nuclear-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 2D 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, Fig7A). However, we did not detect the accumulation of any subcomplex or putative assembly intermediate in our mutants, at least under the conditions tested (Fig7A). To demonstrate that the absence of CCDC56 function was responsible for the mutant CIV



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* (Fig7A, third panel), but not when we overexpressed mitochondrial translation factor B1 (Fig7A, fourth panel). This result indicates that CCDC56 is required for the proper assembly and/ or stability of mitochondrial complex IV in *Drosophila 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, <http://www.flybase.org>). Blast analyses showed that this organization is also present in the other 11 *Drosophila* species for which sequence data is available (data not shown). Northern blot and RACE experiments demonstrate that *ccdc56* 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 genomic approach using *D. melanogaster* and *Anopheles gambiae* transcript annotations and dipteran expressed sequence tags was performed in order to identify transcripts with upstream Open Reading Frames (uORFs). Interestingly, in dipterans conserved uORFs occur preferentially in transcripts encoding mitochondrial proteins and methyltransferases (35). The uORF usually encodes smaller

proteins than those encoded in the main ORF (mORF). 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 if this novel peptide is targeted to the mitochondrial compartment, like mtTFB1, by transfecting HeLa cells with a recombinant d-CCDC56FLAG-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 genomic study, we describe here a new case of a bicistronic transcript in which both proteins have a mitochondrial function. To our knowledge, this is the first demonstration of a eukaryotic bicistron 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* knockout lines via inducing the excision of a P element located in the promoter region of the *ccdc56-mtTFB1* genes. Its imprecise excision removed part of the flanking DNA and therefore generated specific deletions. Transcripts detected by RACE in the *ccdc56*<sup>D6</sup> knockout line lack the first 23 nucleotides and therefore the translation start codon of *ccdc56*, while no *ccdc56* containing transcripts were detected in the *ccdc56*<sup>D11</sup> knockout 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 *ccdc56*<sup>D6</sup> and *ccdc56*<sup>D11</sup> animals. Furthermore, we have demonstrated that both mutant lines retained *mtTFB1*-encoding transcripts and mtTFB1 protein, although in lower levels as compared to 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 (12S 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 and *in-gel* activity assays showed no decrease in the other respiratory chain complex activities in mutant animals, nor 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* knockout 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 CIV deficiency may be responsible for the developmental delay and 100% lethality in the third larval instar observed in *ccdc56* knockout individuals. Mutant wing discs showed a reduced size, a decrease in cell proliferation and an increase in apoptosis levels as compared to 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 and colleagues (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 (reviewed in 41,42). 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 or holo-COX, constitutes the monomeric form of COX that subsequently dimerizes to form the active complex (39). Fully-assembled holo-COX levels were decreased greatly in homozygous *ccdc56*<sup>D11</sup> knockout larvae, as assessed by 2D 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 (43), and reviewed in (44 and 42). 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* knockout 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, increasing number of essential assembly factors for the biogenesis of a functional cytochrome *c* oxidase have been identified in *S. cerevisiae* (4). We found by Blast analysis that CCDC56 homologues are present in metazoans that show high conservation levels. However, no CCDC56 homologue was detected in yeast. The absence of a CCDC56 homologue in yeast might be due to a 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.

## REFERENCES

1. Wallace, D. C., Fan, W., and Procaccio, V. (2010) *Annu Rev Pathol* **5**, 297-348
2. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) *Science* **269**, 1069-1074
3. Szuplewski, S., and Terracol, R. (2001) *Genetics* **158**, 1629-1643
4. Fontanesi, F., Soto, I. C., and Barrientos, A. (2008) *IUBMB Life* **60**, 557-568
5. Galati, D., Srinivasan, S., Raza, H., Prabu, S. K., Hardy, M., Chandran, K., Lopez, M., Kalyanaraman, B., and Avadhani, N. G. (2009) *Biochem J* **420**, 439-449
6. Arnold, S., and Kadenbach, B. (1997) *Eur J Biochem* **249**, 350-354
7. Kadenbach, B., Huttemann, M., Arnold, S., Lee, I., and Bender, E. (2000) *Free Radic Biol Med* **29**, 211-221
8. Barrientos, A., Gouget, K., Horn, D., Soto, I. C., and Fontanesi, F. (2009) *Biochim Biophys Acta* **1793**, 97-107
9. Papadopoulou, L. C., Sue, C. M., Davidson, M. M., Tanji, K., Nishino, I., Sadlock, J. E., Krishna, S., Walker, W., Selby, J., Glerum, D. M., Coster, R. V., Lyon, G., Scalais, E., Lebel, R., Kaplan, P., Shanske, S., De Vivo, D. C., Bonilla, E., Hirano, M., DiMauro, S., and Schon, E. A. (1999) *Nat Genet* **23**, 333-337
10. Valnot, I., von Kleist-Retzow, J. C., Barrientos, A., Gorbatyuk, M., Taanman, J. W., Mehaye, B., Rustin, P., Tzagoloff, A., Munnich, A., and Rotig, A. (2000) *Hum Mol Genet* **9**, 1245-1249
11. Antonicka, H., Mattman, A., Carlson, C. G., Glerum, D. M., Hoffbuhr, K. C., Leary, S. C., Kennaway, N. G., and Shoubridge, E. A. (2003) *Am J Hum Genet* **72**, 101-114
12. Stiburek, L., Vesela, K., Hansikova, H., Hulkova, H., and Zeman, J. (2009) *Am J Physiol Cell Physiol* **296**, C1218-1226
13. Weraarpachai, W., Antonicka, H., Sasarman, F., Seeger, J., Schrank, B., Kolesar, J. E., Lochmuller, H., Chevrette, M., Kaufman, B. A., Horvath, R., and Shoubridge, E. A. (2009) *Nat Genet* **41**, 833-837
14. Xu, F., Addis, J. B., Cameron, J. M., and Robinson, B. H. (2011) *Biochem J*
15. Xu, F., Morin, C., Mitchell, G., Ackerley, C., and Robinson, B. H. (2004) *Biochem J* **382**, 331-336
16. Blumenthal, T. (2004) *Brief Funct Genomic Proteomic* **3**, 199-211
17. Andrews, J., Smith, M., Merakovsky, J., Coulson, M., Hannan, F., and Kelly, L. E. (1996) *Genetics* **143**, 1699-1711
18. Brogna, S., and Ashburner, M. (1997) *EMBO J* **16**, 2023-2031
19. Betran, E., and Ashburner, M. (2000) *Mol Biol Evol* **17**, 1344-1352
20. Estes, P. S., Jackson, T. C., Stimson, D. T., Sanyal, S., Kelly, L. E., and Ramaswami, M. (2003) *Genetics* **165**, 185-196
21. Scarpulla, R. C. (2008) *Physiol Rev* **88**, 611-638
22. Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N. G., and Gustafsson, C. M. (2002) *Nat Genet* **31**, 289-294
23. Seidel-Rogol, B. L., McCulloch, V., and Shadel, G. S. (2003) *Nat Genet* **33**, 23-24
24. Cotney, J., and Shadel, G. S. (2006) *J Mol Evol* **63**, 707-717
25. Matsushima, Y., Adan, C., Garesse, R., and Kaguni, L. S. (2005) *J Biol Chem* **280**, 16815-16820

26. Metodiev, M. D., Lesko, N., Park, C. B., Camara, Y., Shi, Y., Wibom, R., Hultenby, K., Gustafsson, C. M., and Larsson, N. G. (2009) *Cell Metab* **9**, 386-397
27. Engels, W. R., Johnson-Schlitz, D. M., Eggleston, W. B., and Sved, J. (1990) *Cell* **62**, 515-525
28. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res* **25**, 3389-3402
29. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res* **22**, 4673-4680
30. Perez-Carreras, M., Del Hoyo, P., Martin, M. A., Rubio, J. C., Martin, A., Castellano, G., Colina, F., Arenas, J., and Solis-Herruzo, J. A. (2003) *Hepatology* **38**, 999-1007
31. Calvaruso, M. A., Smeitink, J., and Nijtmans, L. (2008) *Methods* **46**, 281-287
32. Pena, P., and Garesse, R. (1993) *Biochem Biophys Res Commun* **195**, 785-791
33. Overbeek, R., Fonstein, M., D'Souza, M., Pusch, G. D., and Maltsev, N. (1999) *Proc Natl Acad Sci U S A* **96**, 2896-2901
34. Stark, A., Lin, M. F., Kheradpour, P., Pedersen, J. S., Parts, L., Carlson, J. W., Crosby, M. A., Rasmussen, M. D., Roy, S., Deoras, A. N., Ruby, J. G., Brennecke, J., Hodges, E., Hinrichs, A. S., Caspi, A., Paten, B., Park, S. W., Han, M. V., Maeder, M. L., Polansky, B. J., Robson, B. E., Aerts, S., van Helden, J., Hassan, B., Gilbert, D. G., Eastman, D. A., Rice, M., Weir, M., Hahn, M. W., Park, Y., Dewey, C. N., Pachter, L., Kent, W. J., Haussler, D., Lai, E. C., Bartel, D. P., Hannon, G. J., Kaufman, T. C., Eisen, M. B., Clark, A. G., Smith, D., Celniker, S. E., Gelbart, W. M., and Kellis, M. (2007) *Nature* **450**, 219-232
35. Hayden, C. A., and Bosco, G. (2008) *BMC Genomics* **9**, 61
36. Brand, A. H., and Perrimon, N. (1993) *Development* **118**, 401-415
37. Adan, C., Matsushima, Y., Hernandez-Sierra, R., Marco-Ferreres, R., Fernandez-Moreno, M. A., Gonzalez-Vioque, E., Calleja, M., Aragon, J. J., Kaguni, L. S., and Garesse, R. (2008) *J Biol Chem* **283**, 12333-12342
38. Maier, D., Farr, C. L., Poeck, B., Alahari, A., Vogel, M., Fischer, S., Kaguni, L. S., and Schneuwly, S. (2001) *Mol Biol Cell* **12**, 821-830
39. Nijtmans, L. G., Taanman, J. W., Muijsers, A. O., Speijer, D., and Van den Bogert, C. (1998) *Eur J Biochem* **254**, 389-394
40. Fornuskova, D., Stiburek, L., Wenchich, L., Vinsova, K., Hansikova, H., and Zeman, J. (2010) *Biochem J* **428**, 363-374
41. Diaz, F. (2010) *Biochim Biophys Acta* **1802**, 100-110
42. Fernandez-Vizarra, E., Tiranti, V., and Zeviani, M. (2009) *Biochim Biophys Acta* **1793**, 200-211
43. Tiranti, V., Corona, P., Greco, M., Taanman, J. W., Carrara, F., Lamantea, E., Nijtmans, L., Uziel, G., and Zeviani, M. (2000) *Hum Mol Genet* **9**, 2733-2742
44. Fontanesi, F., Soto, I. C., Horn, D., and Barrientos, A. (2006) *Am J Physiol Cell Physiol* **291**, C1129-1147
45. Bonnefoy, N., Fiumera, H. L., Dujardin, G., and Fox, T. D. (2009) *Biochim Biophys Acta* **1793**, 60-70

## ACKNOWLEDEMENTS

We thank Verónica Domingo for her excellent technical assistance and Dr. Francisca Diaz for critical reading of the manuscript.

## FOOTNOTES

This work was supported by grants from the Center for Biomedical Research on Rare Diseases (CIBERER), Instituto de Salud Carlos III (grants PI 07/0167 and PI 10/0703 to R.G), the Comunidad de Madrid (grant number GEN-0269/2006 to R.G.), ISCIII-Agencia Laín Entralgo (grant number PI08/0021 to C. U.) and from the National Institutes of Health (grant GM45295 to L.S.K.). S.P. was a postdoctoral fellow from CIBERER.

## FIGURE LEGENDS

**Fig1. 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 (*w<sup>1118</sup>* and *OregonR-C*) and cultured Schneider cell cDNAs. The transcription start points identified are depicted as (+1). The primers R1, R2 and R3 used are represented in A. C) Bicistronic *ccdc56*-*mtTFB1* mRNA detected by Northern blot using 5 mg of RNA from *w<sup>1118</sup>* (C1) and *OregonR-C* (C2) control larvae. The signal detected using a specific *ccdc56* 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*)-CG42630-PA; zebrafish (*Danio rerio*)-A8kB87; western clawed frog (*Xenopus tropicalis*)-A9UM10-1; mouse (*Mus musculus*)-NP\_080894.1; cow (*Bos taurus*)-Q3T0E3; human (*Homo sapiens*)-NP\_001035521.1. Identical residues in all sequences (\*), conserved substitutions (:), and semi-conserved substitutions (.) are noted in the alignment. E) Schematic diagram of the sequences of the human and *Drosophila melanogaster* CCDC56 proteins, showing the putative transmembrane and protein-protein interaction coiled-coil domains.

**Fig2. CCDC56 protein localizes to mitochondria.**

Immunocytochemistry of HeLa cells transfected with recombinant *d*-CCDC56-FLAG. A) MitoTracker staining is shown in red. B) The same cells immunostained for the FLAG epitope (green). The recombinant *Drosophila* protein colocalizes with the MitoTracker dye in the mitochondrial compartment (C). D) Immunoblots of protein extracts (50 µg) from subcellular fractions of control embryos, probed with anti-*Drosophila*-CCDC56 (*d*-CCDC56), anti-porin and anti-GAPDH antibodies. Total extracts, mitochondrial fraction (Mit) and post-mitochondrial supernatant (PMS) are shown.

**Fig3. Molecular characterization of the *ccdc56<sup>D6</sup>* and *ccdc56<sup>D11</sup>* alleles.**

A) Genomic map of the *ccdc56* and *mtTFB1* genes showing the P element insertion (SUPor-P<sup>[kg07792]</sup>, 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 (*w<sup>1118</sup>*) and mutant (*ccdc56<sup>D6</sup>* and *ccdc56<sup>D11</sup>*) homozygous third instar larvae cDNAs identified the transcription start points depicted as (+1). The break points of the deletions generated in this work for the alleles *ccdc56<sup>D6</sup>* and *ccdc56<sup>D11</sup>* are shown in B) and C), respectively. The ratios of the mRNA as determined by 5'-RACE in the clones analyzed are represented (*ccdc56<sup>D6/D6</sup>*, n=8; *ccdc56<sup>D11/D11</sup>*, 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<sup>[kg07792]</sup>, as a negative control. Lane 2: DNA from *w<sup>1118</sup>* control flies. Lane 3: DNA from excised flies without any deletion, used as an additional control. Lane 4: DNA from excised flies of the *ccdc56<sup>D6/D6</sup>* strain, showing a 570 bp deletion. Lane 5: DNA from excised flies of the *ccdc56<sup>D11/D11</sup>* 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 *w<sup>1118</sup>* flies using 18S rRNA as an internal control. The two different Taqman probes used are depicted in B. Control 1: *w<sup>1118</sup>* flies. Control 2: excised flies without any deletion. F) CCDC56 and *mtTFB1* protein levels determined by

immunoblotting of mitochondrial extracts (30 µg) of control and deleted homozygous larvae. Anti VDAC-porin antibody was used as a loading control.

**Fig4. Lack of CCDC56 causes arrest at the third larval stage.**

A) Size comparison of control ( $w^{1118}$ ) 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 15 days AEL larvae, indicating third instar. C) Wing imaginal discs from control ( $w^{1118}$ ) and homozygous  $ccdc56^{D11}$  third instar larvae were dissected and immunostained with anti-phosphohistone-3 (PH-3) antibody. Mutant wing discs showed lower cell proliferation levels as compared to controls. D) Anti-caspase-3 activated antibody was used to detect apoptotic signals in wing imaginal discs. Increased apoptotic levels were observed in homozygous  $ccdc56^{D11}$  flies as compared to control flies. Bar size is 50 µm.

**Table 1. Rescue analysis of  $ccdc56^{D11}/ccdc56^{D11}$  flies.**

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

**Fig5. CCDC56 expression rescues partially the mutant lethality phenotype.**

A) Flies homozygous for the  $ccdc56^{D11}$  allele reach only the pupal stage when they carry on chromosome II the UAS-CCDC56 and the arm-GAL4 transgenes. Larvae and pupae homozygous for the allele  $ccdc56^{D11}$  are of normal size. Larvae and pupae heterozygous for the deletion (genotype  $ccdc56^{D11}/TM6B-Tb$ ) carrying one copy of the *Tb* marker are smaller. B) qRT-PCR of CCDC56, bicistron and mtTFB1 mRNA relative to 18S rRNA, from homozygous  $ccdc56^{D11}$  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 ± SEM 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  $ccdc56^{D11}$  third instar larvae of the genotypes indicated, incubated with polyclonal anti-mtTFB1 and anti-CCDC56 antibodies, and with monoclonal anti VDAC-porin antibody.

**Fig6. *ccd56* knockout flies exhibit an isolated complex IV enzyme deficiency.**

A) Respiratory chain enzyme activities (complexes I, II, III and IV) and citrate synthase activity were measured in mitochondrial extracts obtained from control and *ccdc56* knockout third instar larvae of 15 days AEL. Both mutant alleles showed a severe decrease in complex IV enzyme activity. B) Quantification of relative mtDNA levels by qRT-PCR using ND5 and COXI as mitochondrial gene probes and 18S rRNA as a nuclear gene probe from control,  $ccdc56^{D6/D6}$  and  $ccdc56^{D11/D11}$  third instar larvae of 15 days AEL. C) Steady-state expression levels of representative genes from polycistronic transcripts from mitochondrial RNA were measured by qRT-PCR from  $ccdc56^{D6/D6}$  and  $ccdc56^{D11/D11}$  third instar larvae of 15 days AEL, and are shown relative to the levels found in control larvae, after normalization to 18S rRNA levels. Control larvae: genotype  $w^{1118}$ ; D6/D6:  $ccdc56^{D6/D6}$  and D11/D11:  $ccdc56^{D11/D11}$ . Data shown in A, B and C represent the mean ± SEM of at least three independent determinations; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; ANOVA.

**Fig7. Loss of *ccdc56* induces a complex IV assembly defect.**

A) 2D-BN/ SDS-PAGE analysis of mitochondrial extracts immunoblotted with an antibody against the mitochondrial-encoded subunit mt-CO3 of the cytochrome *c* oxidase complex. Mitochondria-enriched extracts were prepared from third instar larvae of the following genotypes: 1-control  $w^{1118}$ ; 2-mutant  $ccdc56^{D11/D11}$ ; 3-mutant ubiquitously expressing the UAS-CCDC56 transgene *arm-GAL4/UAS-CCDC56*;  $ccdc56^{D11/D11}$ ; and 4-mutant larvae expressing the UAS-*mtTFB1* transgene under the same condition (*arm-*

*GAL4/UAS-mtTFB1;ccdc56<sup>D11/D11</sup>*). The lack of fully-assembled holo-COX exhibited by the *ccdc56<sup>D11/D11</sup>* mutant is rescued partially by overexpression of the *UAS-CCDC56* transgene. S4, fully assembled complex IV. The previously identified mt-CO3-containing COX subcomplex S3 is indicated. B) First dimension of a duplicate BN-PAGE of mitochondrial extracts incubated with a polyclonal antibody against complex V as a loading control for the 2D- BN/ SDS-PAGE shown in A. C) Complex IV enzyme activity measured in mitochondrial extracts from homozygous *ccdc56<sup>D11/D11</sup>* third instar larvae carrying the indicated constructs on chromosome II. Data were normalized to control larvae (*w<sup>1118</sup>*) and represent the mean SEM, n=3; \*p<0.05; *Student's t-test*.



Figure 1

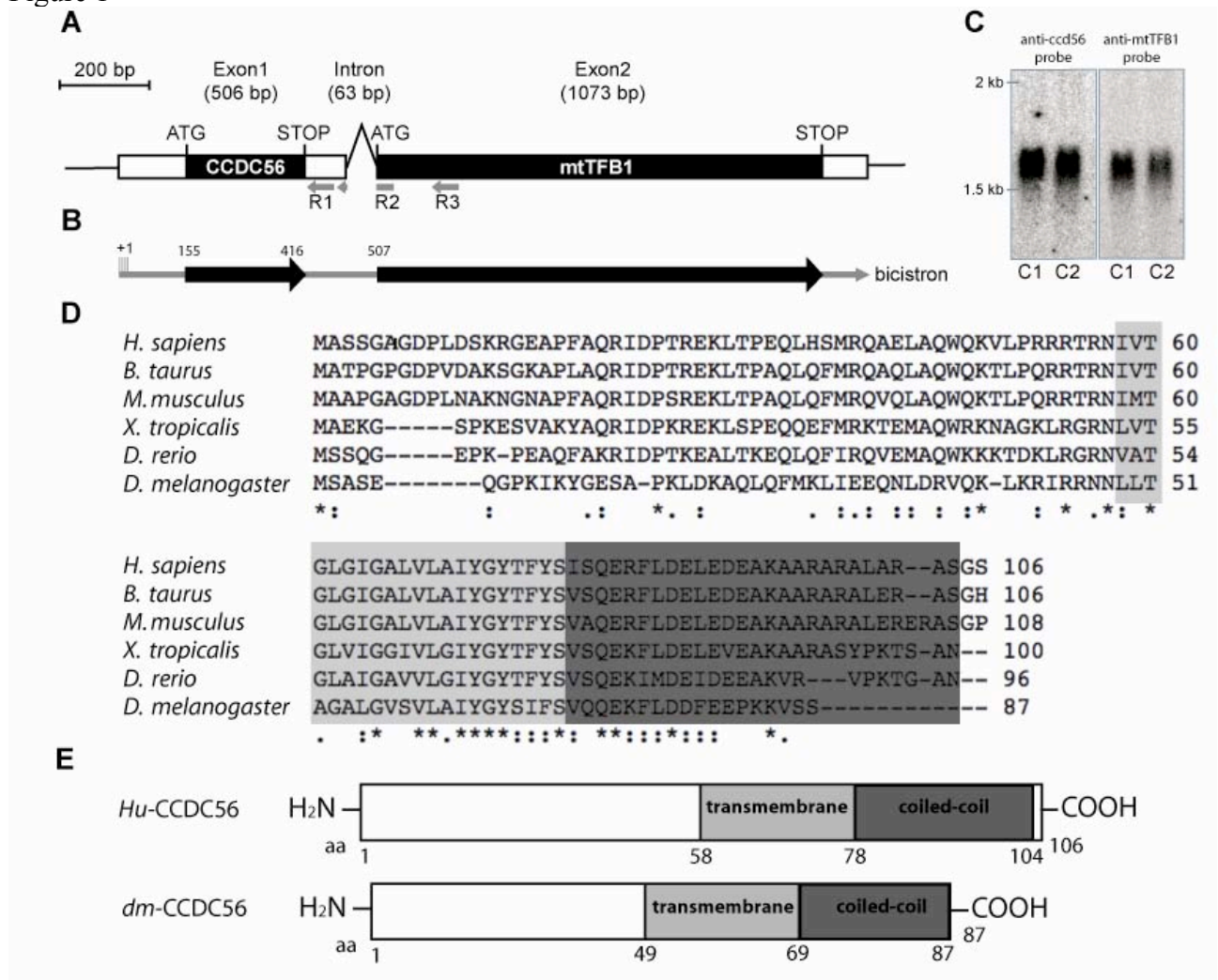


Figure 2

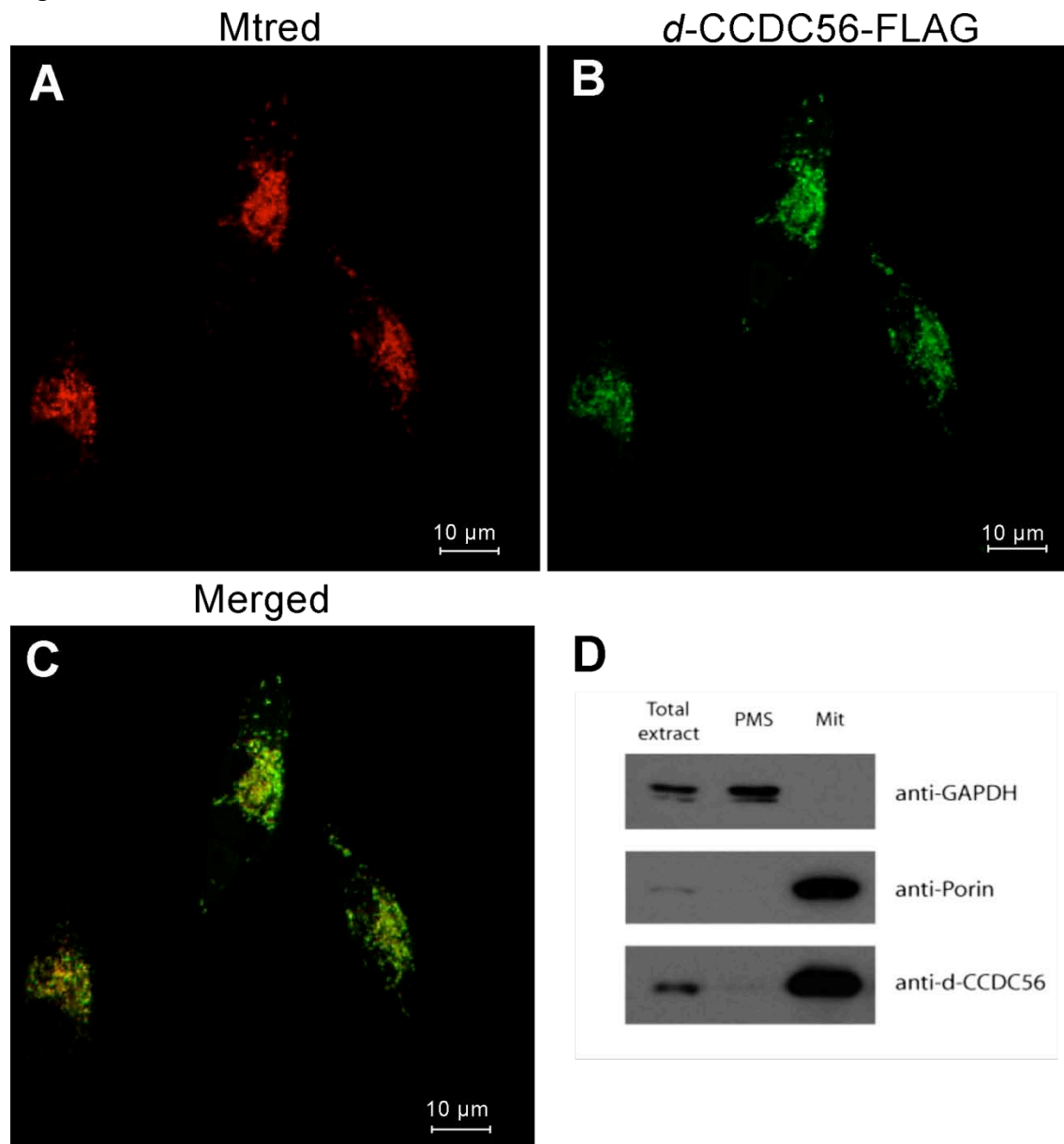


Figure 3

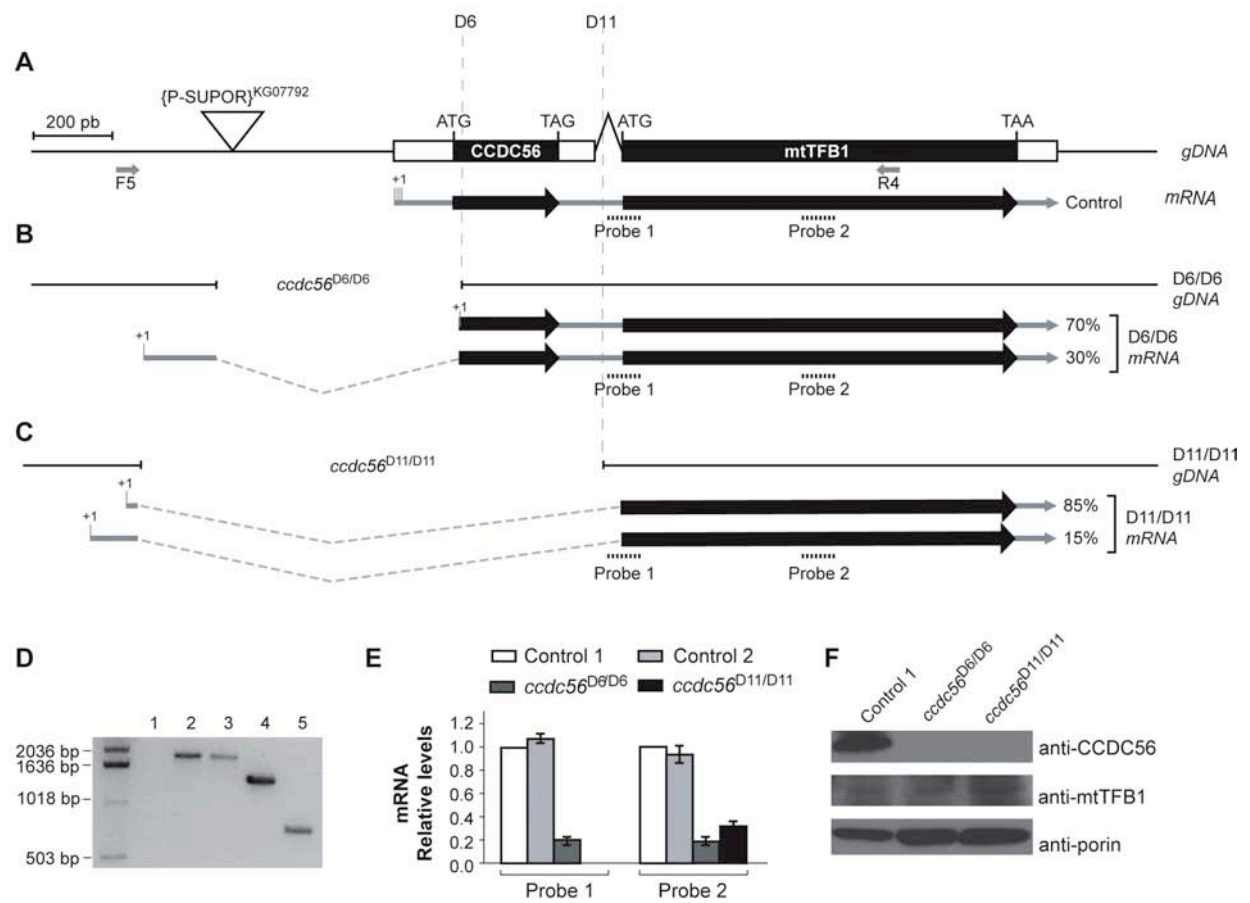


Figure 4

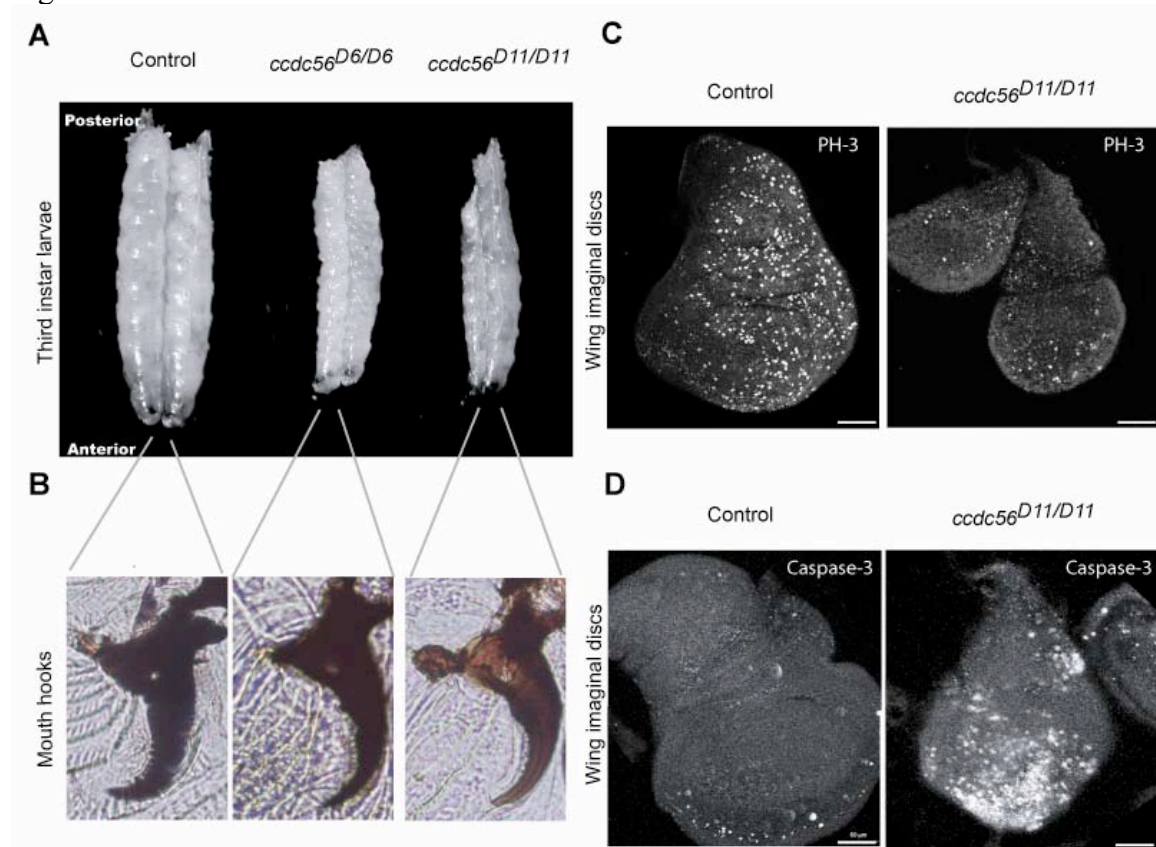


Table 1

**TABLE 1**  
**Rescue analysis of *ccdc56*<sup>D11</sup>/*ccdc56*<sup>D11</sup> flies**

Genotype Chr II	N° of <i>ccdc56</i> <sup>D11</sup> /TM6B-Tb pupae scored	N° of <i>ccdc56</i> <sup>D11</sup> / <i>ccdc56</i> <sup>D11</sup> pupae scored	N° of Total pupae scored	Mean (%) <i>ccdc56</i> <sup>D11</sup> / <i>ccdc56</i> <sup>D11</sup> pupae scored/expected <sup>a</sup> ±SEM
+/ +	1306	0	1306	0
+/ <i>arm-GAL4</i>	1527	0	1527	0
<i>UAS-CCDC56-1</i> / +	1622	0	1622	0
<i>UAS-CCDC56-1</i> / <i>arm-GAL4</i>	1823	487	2310	62.8 ± 1.8
<i>UAS-CCDC56-2</i> / +	1786	0	1786	0
<i>UAS-CCDC56-2</i> / <i>arm-GAL4</i>	1502	356	1858	55.9 ± 4.8
<i>UAS-mtTFB1-1</i> / +	2039	0	2039	0
<i>UAS-mtTFB1-1</i> / <i>arm-GAL4</i>	1984	0	1984	0
<i>UAS-mtTFB1-2</i> / +	1629	0	1629	0
<i>UAS-mtTFB1-2</i> / <i>arm-GAL4</i>	1626	0	1626	0

Figure 5

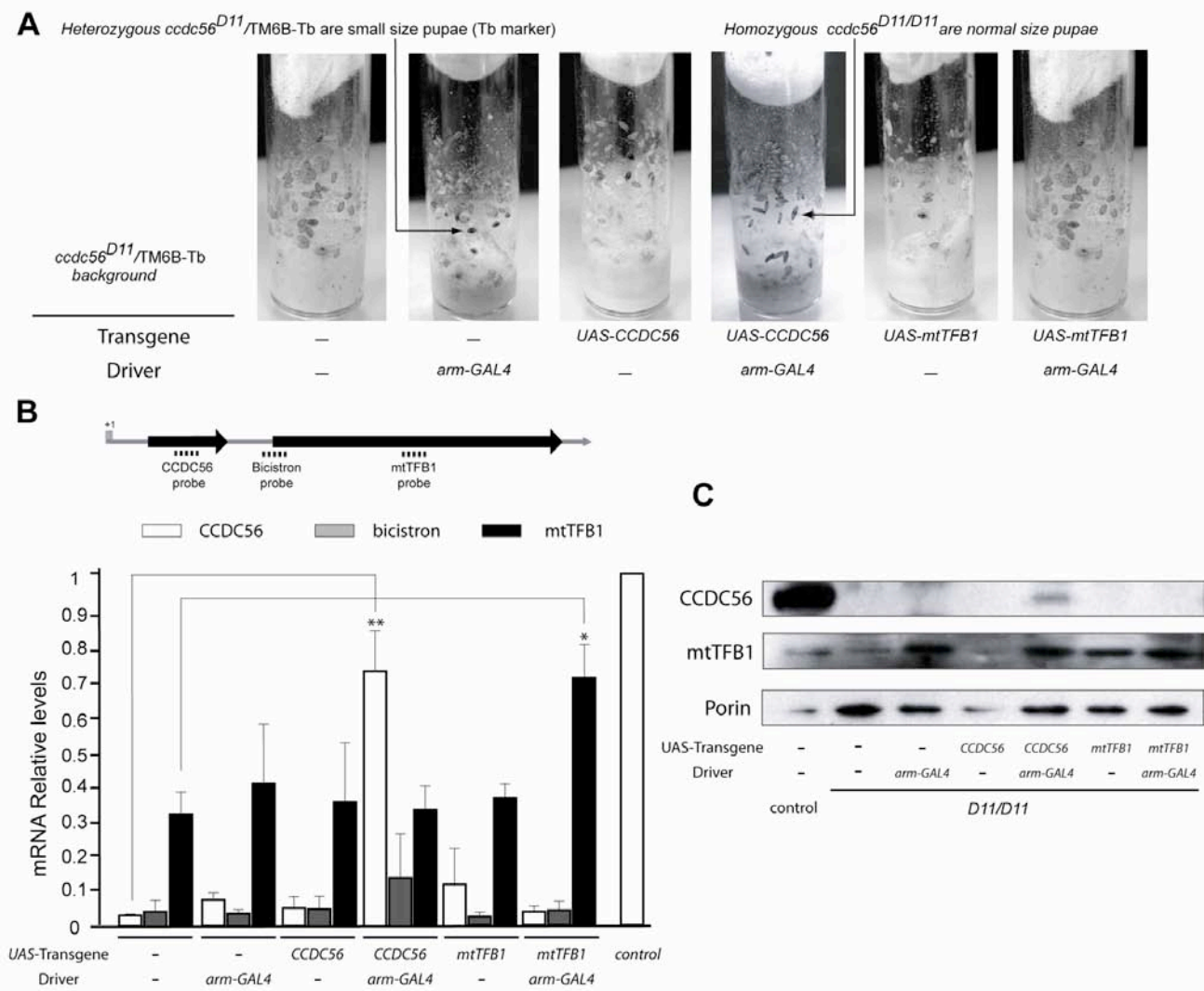
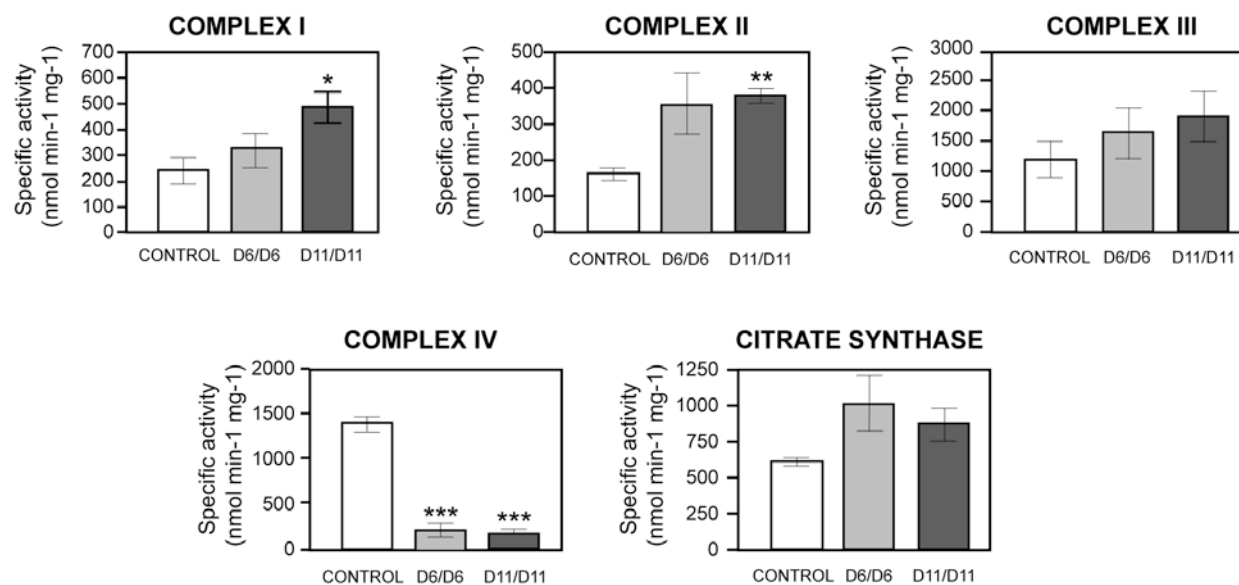
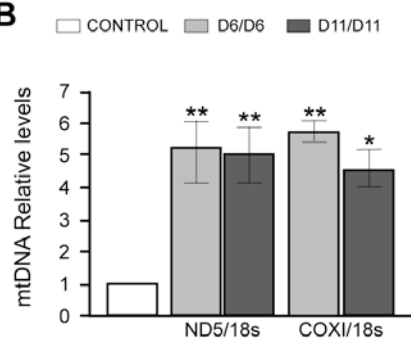


Figure 6

**A**



**B**



**C**

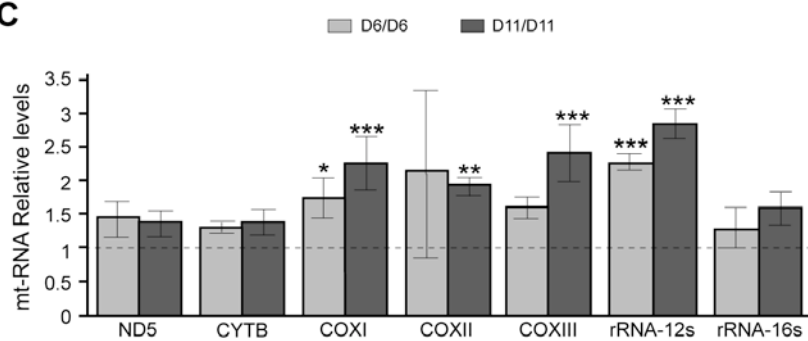


Figure 7

