Supplementary Table S1. Detailed information about the strains used in this study.

Strain name	Genotype	Stock Center
COX4L-RNAi /CG10396-RNAi (ID # 1482)	*w^{1118}; P[GD414]v1482*	VDRC
COX4L-RNAi /CG10396-RNAi (ID # 106700)	*w^{1118}; P[KK102531]v106700*	VDRC
Nos Cas9 attp2	*y,sc,v; +/+; nos-Cas9*	Rainbow Transgenic Flies, Inc.
Actin5C-Gal4 (ID # 4414)	*y[1] w[*]; P{w[+mC]=Act5C-GAL4}25FO1/CyO, y[+]*	BDSC
bam-Gal4	*y[1] w[*] P{w[+mC]=bam-GAL4:VP16}1*	BDSC
nos-Gal4 (ID # 25394)	*w[*]; Pen[D14]/CyO; P{w[+mC]=GAL4-nos.NGT}9/TM6B, Tb[+]*	BDSC
w^{1118} /GD control (ID # 60000)	*w[1118]*	VDRC
w^{1118}/KK control (ID # 60100)	*y,w[1118];P{attP,y[+],w[3`]}*	VDRC

Supplementary Table S2. gRNA primers and homologous arm gene blocks (5’-3’) designed to produce the *COX4L* knockout and primers to confirm the *COX4L* knockout via PCR.

COX4L guide 1 sense	CTTCGTGTAATTATGCGCAAGCACT
COX4L guide 1 antisense	AAACAGTGCTTGCGCATAATTACAC
COX4L homologous arm 1 *Eco*RI	GGGTGTCGCCCTTCGCTGAAGCAGGTGGAATgcttttcgctggcc atagctctcgtggcccttgcagccactctggttcgtgccaatatagatgataacctgccaa ttaacactgagggacggattgattttgatttcggcttcgaagaggcaatttggggaaaaa accgtaaaaataggaaatatctaaagaatagctcgacaatttttcacaaattacaaattt aattattaaataattatttggaaagtttttaaaatttttgttttcggaattgttttatttttgtgttttttttt ttcatgatatttacttttaagagattggcaaatgcttcattctctaagtagagcgagattgtctt taatgtcttatattttctaaagtatagctttttttaaaattcttaagggtgggccaacaatgttatt gcgatttaaaaatttttgaaaaaagtcaactagttgattcttaaactttatcaaaatttcagat attgaaaactggacgtgggcaaaaaaaataattattgggcaaacagttctagatttcaa aaattcgatttttccgaacccagcttctttgagctgacatgacagccatttttaaaaatgtttg ttttttttttgtgacaaaaaatttgatcttcataatttttgccacgccttaaacaatttttaagaa gaagtaaaattttcagactatcttagtgctcaacgaagagtgcaattcagaacttaaaaa gtacatctagtttgtagataaggaaactgtcatattttttttgtattcaacaaacagactaga gaaatttcattttcattcgacacgagcaacacaactgtcgaatttccggatgaagtaaaa aacaaaaaattgaaaagcgagtataaaataaaatacactcaaggtacagttacgacc aagtAATTCTTGCATGCTAGCGGCCGCGGACATAT
COX4L guide 2 sense	CTTCGTTCTCTCGGTAGCACCATT
COX4L guide 2 antisense	AAACAATGGTGCTACCGAGAGAAC

COX4L homologous arm 2 *Xho*I	TGCATAAGGCGCGCCTAGGCCTTCTGCAGCggtgctaccgagag aacaagtggaagtagcacatcaacacgatgattttccgtaaactatgtacagaaacgta actagcaaaatacaattcaacagcaaagtcgcttgccatttgatcgttacgtgctgaatc gggcaaatacccaatatctatagatttttgtgtctctagctgtgtaactcgactatagcatttc ctcccgtttgaaattagggtgtgtatgtaaattctcagacacaacttaatttagtgtaatttta gtccacgatagatatgttaagcattgaaatcgtgtcctgtgttcctttgactagtacacgtac actgcgcgtcatcagattagcgcctccctgtatgccaccgtttcatcttatgatctgtattttc cattgcacgaaaatctatcaatgttattgtttttgttcactgatattccctctctctttgagaata aaaaagaggttgagagaagaacagttatctcttttattctgctttgtgttaactttggcgcaa aattgaacacgtgttttgcatcatcagattagcgcccccagttttgaatatcgtccataattt gaaaggtaggacaacaaaattttattaaaaacaaggaatcttatagaaaaaactataa ttgtggcaataaccgtgtcgttaccgcggcagattgcaggtccatacttcgaattgcttcca attcccacgactcctccgccaaaattcgagaaaaaagtggtataagcgcgagcaaatc gacgattccaagggtgacttagaaagctaaacatttaaagcacaacgaactaacGC TCGAGGCTCTTCCGTCAATCGAGTTCAAG
Forward primer to confirm the *COX4L* knockout	GTACACTGCCGTCGAAATGAG
Reverse primer to confirm the *COX4L* knockout	GGGTTCATTTGCAGCTGGATG

Supplementary Table S3. gRNA primers and homologous arm gene blocks (5'-3') designed to produce the *COX4* knockout and primers to confirm the *COX4* knock-out via PCR.

COX4 guide 1 sense	CTTCGTATATTCAATCGCTAGCAAT
COX4 guide 1 antisense	AAACATTGCTAGCGATTGAATATAC
COX4 homologous arm 1 *Eco*RI	GGGTGTCGCCCTTCGCTGAAGCAGGTGGAATgcgcacttgttaaaaatgcaaattgtcctagccacaaactgaacctgataacgaagcactaccatcccaaaaagcaaggaccccggctgcgtttccgaaaaacccaaatcttgagaaaaggaaacttcactaacatgaaatgttacagagataagacgagtgaattcatttttgtacatatagatgaaaactaaccccaagtccccccctatgcttaagtgtgaatctgaccgacggtctagactttttatacccttttatcccagcccagagtcggcggatcatatgaattaaatcgcaactaatacctaactctactgtaaatgtagctataagtttttgctactaccaatttacaagatgtatagttgaaggaaaaagctttcactttagtttaattatttgcgtaattatcattaaatattgttacaaacatattgcatatttataaagcccaataaagcaaaataccattaacatatttattatgccaagcggcgcttaaacacaggaatacgcgaaagtgaatacgtaatgccccatcctcgataaaatagctaaatttccgaactccagatccctatcgaaagataatcttatgtaatacaaactcaagcagcaaactatatgatacgataaagcaaattatgtgtctctgtctaatttaaatgtgtgcatttatgagatatgacatcaaatcaaaataaaatataatatcgaacaataattgttttgttgttgtttctcttctcgcggacagactacaaaaagaatgtgaaataccaaaccaaatatttcaccatgctgcgaaataaaataaaacataatagttgcccttatttgctgtttctttattttaatatatttagatttaaataccccgccgaattccgattAATTCTTGCATGCTAGCGGCCGCGGACATAT
COX4 guide 2 sense	CTTCGCTCTTAAAACGGATCATTG

COX4 guide 2 antisense	AAACCAATGATCCGTTTTAAGAGC
COX4 homologous arm 2 *Xho*I	TGCATAAGGCGCGCCTAGGCCTTCTGCAGCtgatccgttttaagagcaatgattgttattcgatttattaacagaccttttagtgtgtagattacaaacttttcgttactaaaatcgccacaggcatagccaatttaattaccacttagtgagaccttttagcactcttatgtctcatgttccggctgttcgatttttgaacctcatgaacaaggatctgcaaggcttccaaggcatttgtgttcttggcaaattccctttcaaacatgcggccgaatcttaagatcaatggtttcagttccggcgttatcattgctttctctttttcggtcctaaaaaggcggatttcaaaggcattgccagacatataggcgaacactacttacacagctcccttttttatcacctctttcaggatctggacaaccgtttgaatgtccccaaataatctgtacttgtctttggtagaaatttcaccgtcaaagaataacttcagttcgttgtcagttatattcttctttcgaccccattcctccagctctgccgtctctttttggtaggcgctccgagctctgctgaaaagaagtcaggcattccttgatctcagagcattcatacttgatgcagatgatgcacctacctccagttcgaaatatccctagctgtctgcttcatcttttttaaatgtcctgatctgtgtttgaaagtcttctcttctttctcctttgcaactgtcgcctttggttcggcatctcggttaaagctattgaaagcgaaaggttggtgccattcgaaagtgtatatatccttcagctcgatcgaatctgtatcttactgtttgtgccaatcgtatccatcgtatggaggcctgtgcagattgtgtgctcggagatgacgctgtctttccatctgggtgtggttctggttaccGCTCGAGGCTCTTCCGTCAATCGAGTTCAAG
Forward primer to confirm the *COX4* knockout	ACTACTCAACAGTGCTGTGCT
Reverse primer to confirm the *COX4* knockout	GTGTAGTCCTCGTTGTGGGA

Supplementary Table S4. Evolutionary rate covariation (ERC) values calculated using the ERC Analysis Web server from Pittsburg University (https://csb.pitt.edu/erc_analysis) [1-3]. The presence of testis-enriched N-mt gene duplicates involved in OXPHOS complexes is retrieved from the *Drosophila* sperm proteome project (DmSP-II) [4].

Duplicate Gene ID	Duplicate Gene Name	DSP	DSP Absent (Query COX4)	DSP Present (Query COX4)	DSP Absent (Query COX4L)	DSP Present (Query COX4L)
CG6485	*ND-24L*	DmSP-II	-	0.091	-	0.371
CG8102	*ND-51L2*	DmSP-I	-	-0.289	-	0.424
CG2014	*ND-20L*	X	-0.073	-	0.11	-
CG11913	*ND-49L*	DmSP-I	-	0.176	-	0.023
CG5718	*SdhAL*	DmSP-I, DmSP-II	-	0.151	-	0.554
CG14508	*Cyt-c1L*	DmSP-I, DmSP-II	-	0.285	-	0.275
CG10396	*COX4L*	DmSP-I	-	0.348	-	N/A
CG5389	*ATPsynbetaL*	DmSP-I, DmSP-II	-	0.395	-	0
CG18418	*CG18418*	X	0.496	-	0.4	-
CG4701	*CG4701*	X	0.612	-	-0.056	-
CG14740	*CG14740*	DmSP-I, DmSP-II	-	0.303	-	0.829
CG6255	*Scsα2*	DmSP-I, DmSP-II	-	0.517	-	0.215
CG33791	*CG33791*	DmSP-II	-	-0.08	-	0.527
CG33092	*P5CDh2*	X	0.553	-	0.343	-
CG11401	*Trxr-2*	X	0.59	-	0.475	-
CG9920	*BEST:GH20473*	X	-0.046	-	0.166	-
Average			0.354	0.1897	0.24	0.357

Supplementary Table S5. One-ratio branch model analysis with a null model assuming that each respective group of sequences is evolving at the same rate (one-ratio model) in addition to an alternative model in which the *d*N/*d*S ratio was fixed to *d*N/*d*S = 1, conducted for *COX4* (A) and *COX4L* (B). **C)** Two-ratio branch model analysis for the comparison of *COX4* and *COX4L* evolution under different evolutionary constraints. Significantly higher rate is shown in red.

A.

COX4	Branch model- One Ratio	omega (dN/dS)	0.06967
		lnL	-3785.3
		np	41
	omega =1	omega (dN/dS)	1
		lnL	-4273.55
		np	40
	*P*value:	0	

B.

COX4L	Branch model- One Ratio	omega (dN/dS)	0.10732
		lnL	-4755.83
		np	43
	omega =1	omega (dN/dS)	1
		lnL	-5221.51
		np	42
	*P*value:	0	

C.

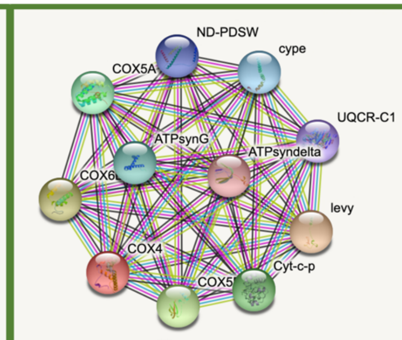
	logL; 1 rate	**Single rate**	**logL; 2 rates**	**COX4 rate**	**COX4L rate**	**2deltaL~X2**	**d.f.**	***P***
COX4-COX4L	-4614.928441	0.1006	-4606.91099	0.0606	0.125	16.034902	1	0.0001

Supplementary Table S6. Physical protein interaction analysis of *COX4L* with STRING (V 11.5)

COX4 — *Cytochrome-c oxidase activity. It is involved in the biological process described with: negative regulation of neuroblast proliferation; cell proliferation; mitochondrial electron transport, cytochrome c to oxygen; mitotic cell cycle; Golgi organization (182 aa)*

Predicted Functional Partners:

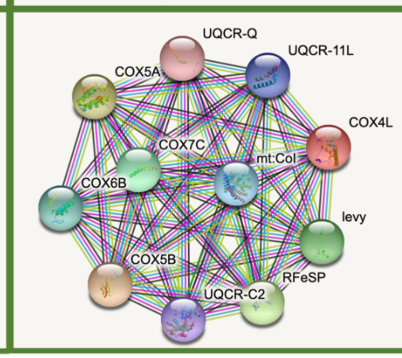
Protein	Description	Neighborhood	Gene Fusion	Cooccurence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
levy	*Levy, isoform A; Enzyme regulator activity; cytochrome-c oxidase activity. It is involved in the biological process described wit...*				●	●	●	●		0.999
COX6B	*Cytochrome c oxidase subunit; This protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the term...*				●	●	●	●		0.999
COX5B	*Cytochrome-c oxidase activity. It is involved in the biological process described with: mitochondrial electron transport, cytoch...*				●	●	●	●		0.999
COX5A	*Cytochrome c oxidase subunit 5A, mitochondrial; This is the heme A-containing chain of cytochrome c oxidase, the terminal o...*				●	●	●	●		0.999
ATPsynG	*Hydrogen-exporting ATPase activity, phosphorylative mechanism. It is involved in the biological process described with: proto...*				●			●		0.999
cype	*Cyclope (Cype) is a cytochrome c oxidase subunit VIc homolog acting as an enhancer of dpp pathway phenotypes. Cype is in...*				●	●	●	●		0.999
UQCR-C1	*Ubiquinol-cytochrome-c reductase activity; metal ion binding; metalloendopeptidase activity. It is involved in the biological pro...*				●	●	●	●		0.995
Cyt-c1	*Cytochrome c1, isoform A; Electron transporter, transferring electrons within CoQH2-cytochrome c reductase complex activit...*				●	●	●	●		0.993
COX7A	*Cytochrome c oxidase subunit 7A, mitochondrial; This protein is one of the nuclear-coded polypeptide chains of cytochrome ...*				●	●	●	●		0.993
RFeSP	*Rieske iron-sulfur protein*				●	●	●	●		0.991



COX4L — *Cytochrome c oxidase subunit 4-like, isoform a; Cytochrome-c oxidase activity. It is involved in the biological process described with: mitochondrial electron transport, cytochrome c to oxygen (176 aa)*

Predicted Functional Partners:

Protein	Description	Neighborhood	Gene Fusion	Cooccurence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
COX5B	*Cytochrome c oxidase subunit 5b, isoform a; Cytochrome-c oxidase activity. It is involved in the biological process described...*				●	●	●	●		0.994
COX5A	*Cytochrome c oxidase subunit 5A, mitochondrial; This is the heme A-containing chain of cytochrome c oxidase, the terminal ...*				●	●	●	●		0.993
RFeSP	*Ubiquinol-cytochrome c reductase iron-sulfur subunit; Component of the ubiquinol-cytochrome c reductase complex (compl...*				●	●	●	●		0.991
levy	*Levy, isoform A; Enzyme regulator activity; cytochrome-c oxidase activity. It is involved in the biological process described wi...*				●	●	●	●		0.988
COX7C	*Cytochrome c oxidase subunit 7c, isoform a; Cytochrome-c oxidase activity. It is involved in the biological process described...*				●	●	●	●		0.988
COX6B	*Cytochrome c oxidase subunit 6b, isoform d; Cytochrome c oxidase subunit; This protein is one of the nuclear-coded polype...*				●	●	●	●		0.987
mt:CoI	*Cytochrome c oxidase subunit 1; Cytochrome c oxidase is the component of the respiratory chain that catalyzes the reductio...*				●	●	●	●		0.987
UQCR-11L	*Ubiquinol-cytochrome c reductase 11 kda subunit-like, isoform b; Cytochrome b-c1 complex subunit 6; This is a component ...*				●	●	●	●		0.985
UQCR-C2	*Ubiquinol-cytochrome c reductase core protein 2, isoform a; Ubiquinol-cytochrome-c reductase activity; metal ion binding. It i...*				●	●	●	●		0.984
UQCR-Q	*Ubiquinol-cytochrome c reductase ubiquinone-binding protein, isoform a; Ubiquinone binding; ubiquinol-cytochrome-c reduct...*				●	●	●	●		0.983



Supplementary Table S7. Average values of single cell expression of COX4 and COX4L from the spermatogonia stage to late primary spermatocytes [5].

FBgn	Gene Symbol	G (Spermatogonia)	E1° (Early Primary Spermatocytes)	M1° (Middle Primary Spermatocytes)	L1° (Late Primary Spermatocytes)
FBgn0032833	***COX4***	619.63	296.1673225	213.5008964	149.4138946
FBgn0033020	***COX4L***	167.36	1214.38135	1423.794829	1959.815878

Supplementary Table S8. Average values (± SE) of viability results of *COX4L* knockdown in the soma with the *Actin5c-Gal4* driver at 25°C and 27°C.

Crosses	Average Number of Progeny at 25 °C	Average Number of Progeny at 27 °C
♀COX4L-KK x ♂Actin5c-Gal4	91.3 ± 9.9	101.3 ± 1.7
♂COX4L-KK x ♀Actin5c-Gal4	86.7 ± 2.4	100.7 ± 6.1
♀COX4L-KK x ♂w^{1118}	80.7 ± 11.7	85.7 ± 17.8
♂COX4L-KK x ♀w^{1118}	65.0 ± 23.6	87.3 ± 15.2
♀Actin5c-Gal4 x ♂w^{1118}	78.7 ± 2.9	79.3 ± 5.0
♂Actin5c-Gal4 x ♀w^{1118}	80.0 ± 7.4	82.0 ± 7.9
♀COX4L-GD x ♂Actin5c-Gal4	80.0 ± 8.3	64.7 ± 3.5
♂COX4L-GD x ♀Actin5c-Gal4	59.3 ± 14.8	72.7 ± 2.2
♀COX4L-GD x ♂w^{1118}	85.7 ± 12.4	66.3 ± 5.7
♂COX4L-GD x ♀w^{1118}	70.7 ± 1.9	58.3 ± 9.3
♀Actin5c-Gal4 x ♂w^{1118}	67.3 ± 2.6	62.0 ± 9.3
♂Actin5c-Gal4 x ♀w^{1118}	72.7 ± 1.5	67.3 ± 6.3

Supplementary Table S9. Average values (± SE) of fertility results of *COX4L* knockdown in the germline with the *bam-Gal4* driver at 25°C and 27°C.

Crosses	**Average Number of Progeny at 25 °C**	**Average Number of Progeny at 27 °C**
♀ [♀KK x ♂Gal4](27c) x ♂W1118(25c)	69.3± 4.9	80.3± 1.2
♀[♀KK x ♂W1118] (27c) x♂W1118(25c)	57.0 ± 3.7	68.0± 1.4
♂ [♀KK x ♂Gal4](27c) x ♀W1118(25c)	44.7± 8.6	51.7± 2.9
♂[♀KK x ♂W1118] (27c) x♀W1118(25c)	72.7± 4.2	71.0± 8.1
♀[♂GD x ♀Gal4] (27c) X ♂W1118(25c)	69.0± 4.7	80.3± 1.6
♀[♂GD x ♀W1118](27c) X ♂W1118(25c)	60.3± 3.5	65.7± 2.4
♂[♂GD x ♀Gal4] (27c) X ♀W1118(25c)	47.0± 2.1	59.7± 4.1
♂[♂GD x ♀W1118] (27c) X ♀W1118(25c)	62.3± 3.3	74.0± 1.9

Supplementary Table S10. Average values (± SE) of viability results of *COX4L* knockout strain at 25°C.

Crosses	Average Number of KO/KO Progeny at 25 °C	Average Number of KO/CyO Progeny at 25 °C
♀KO/CyO x ♂KO/CyO(25c)	23.3 ± 1.8	39.7 ± 1.2
	Average Number of +/+ Progeny at 25 °C	**Average Number of +/CyO Progeny at 25 °C**
♀+/CyO x ♂+/CyO(25c)	24.3 ± 1.2	40.0 ± 1.0

Supplementary Table S11. Average values (± SE) of viability results of *COX4* and *COX4L* overexpression or ectopic expression in the soma and germline with the *Act-5C-Gal4* and *bam-Gal4* drivers at 25°C.

Crosses	**Progeny without TM3**	**Progeny with TM3**
COX4-ORF x *bam-Gal4*	42 ± 3.6	48.7 ± 2.3
COX4L-ORF x *bam-Gal4*	50.3 ± 3.4	47.3 ± 1.5
w^{1118} x *bam-Gal4*	48 ± 5.8	46.7 ± 2
Crosses	**Progeny without CyO**	**Progeny with CyO**
COX4-ORF x *Act-5C-Gal4*	47 ± 0.6	44 ± 2
COX4L-ORF x *Act-5C-Gal4*	0	48.3 ± 1.8
w^{1118} x *Act-5C-Gal4*	49.3 ± 2.8	44.3 ± 4.4

Supplementary Table S12. Average values (± SE) of fertility results of *COX4* and *COX4L* overexpression in the germline with the *bam-Gal4* driver at 25°C.

Crosses	Average Number of Progeny at 25 °C
♀ *COX4*-ORF/bam-Gal4 x ♂ w^{1118}	25 ± 1.73
♂ *COX4*-ORF/bam-Gal4 x ♀ w^{1118}	24.7 ± 3.28
♀ *COX4L*-ORF/*bam-Gal4* x ♂ w^{1118}	24 ± 5.55
♂ *COX4L*-ORF/*bam-Gal4* x ♀ w^{1118}	12 ± 0.57
♀ w^{1118}/ *bam-Gal4* x ♂ w^{1118}	25.3 ± 1.2
♂ w^{1118}/ *bam-Gal4* x ♀ w^{1118}	23.3 ± 3.18
Crosses	**Average Number of Progeny at 25 °C**
♀ *COX4*-ORF/ *Act-Gal4* x ♂ w^{1118}	25 ± 1.73
♂ *COX4*-ORF/ *Act-Gal4* x ♀ w^{1118}	25.3 ± 4.05
♀ *COX4L*-ORF/ *Act-Gal4* x ♂ w^{1118}	27.3 ± 2.02
♂ *COX4L*-ORF/ *Act-Gal4* x ♀ w^{1118}	29.3 ± 1.45
♀ w^{1118}/ *Act-Gal4* x ♂ w^{1118}	25.3 ± 3.84
♂ w^{1118}/ *Act-Gal4* x ♀ w^{1118}	26.3 ± 1.2

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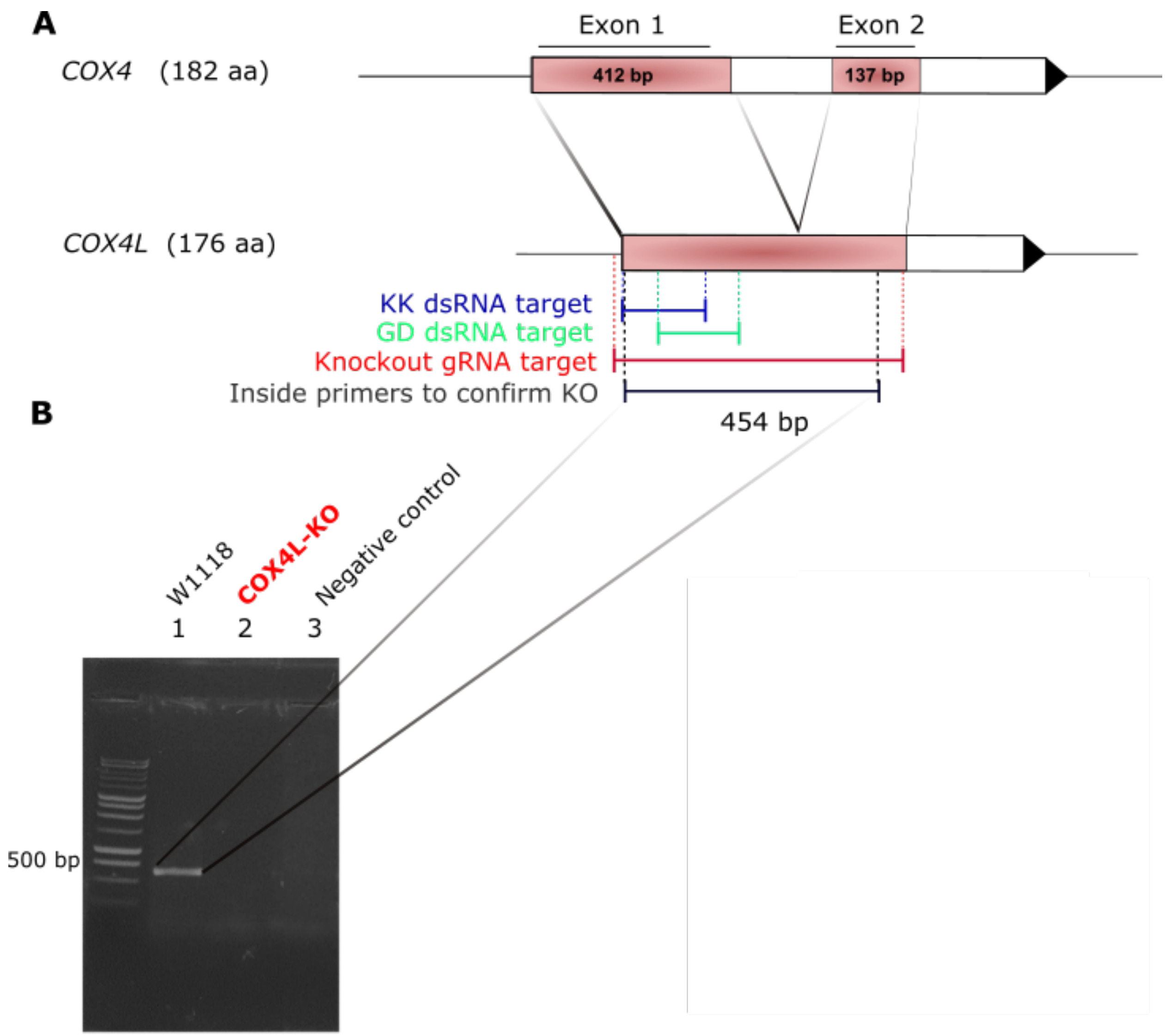
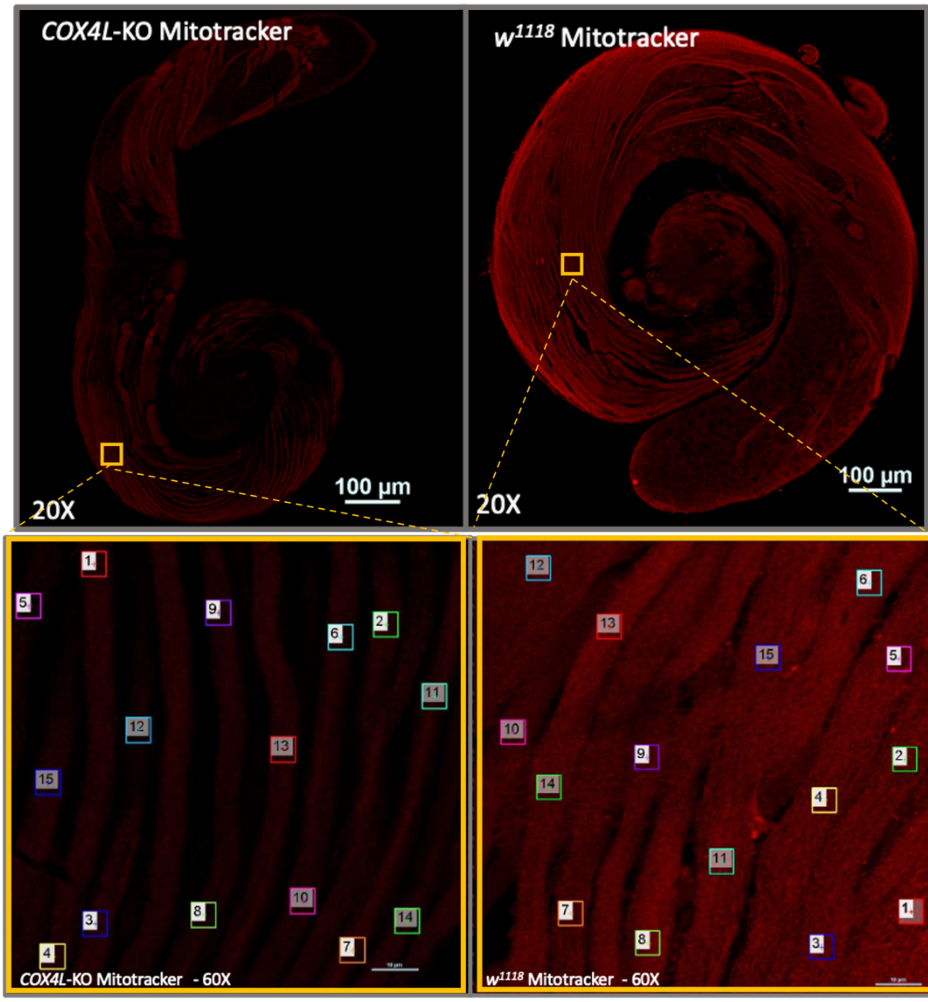


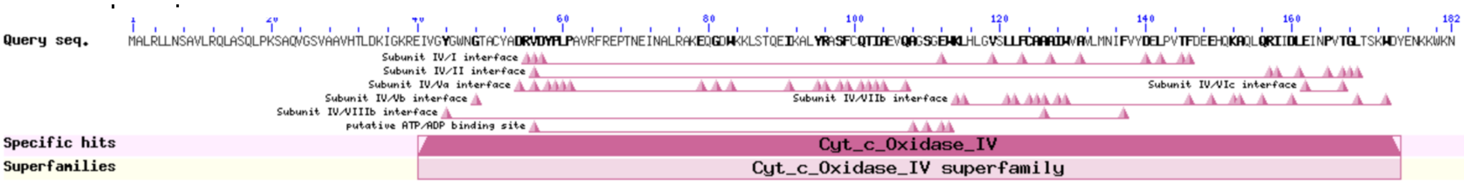
Supplementary Figure S1. (A) *COX4* and *COX4L* gene structures. *COX4L* is an RNA-mediated duplication of *COX4*, which is only six aa shorter than its parental protein. The regions of dsRNA expressed in the RNAi knockdowns using the KK and GD lines are shown. The *COX4L*-KO targeted region is shown. The location of the primers for PCR within *COX4L* is depicted. (B) The lack of a PCR amplification for *COX4L*-KO homozygotes is shown, confirming the removal of *COX4L* from the genome.



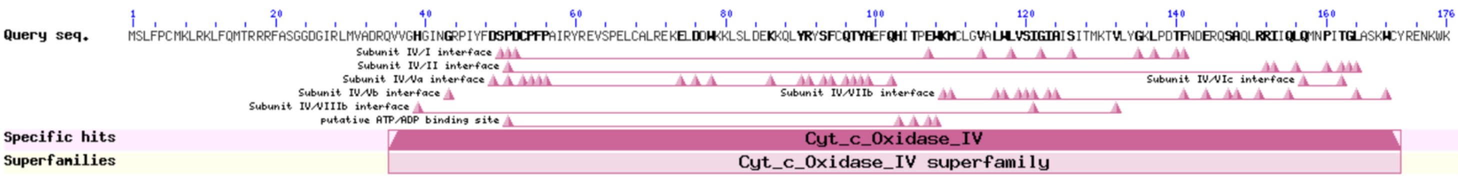
	W1118	COX4L-KO
1	1374.847	401.1494
2	1233.292	464.0166
3	1317.888	435.0584
4	1173.344	411.9317
5	1138.54	220.4361
6	1414.348	428.3314
7	955.9697	457.9955
8	1356.744	298.4862
9	976.3196	365.6724
10	744.9306	357.4796
11	1309.228	416.8616
12	950.3059	201.5548
13	1353.45	429.9167
14	1020.872	325.0104
15	1183.272	386.3094
Mean	1166.890053	373.347347
P-value	1.49139E-10	

Supplementary Figure S2. *COX4L*-KO and w^{1118} sperm bundles stained with MitoTracker™ Deep Red FM are shown. The confocal microscope at UT Arlington (Nikon Eclipse Ti2 laser scanning confocal microscope) was used for imaging. NIS-Elements imaging software (version 5.20.00) was used for image visualization. The fluorescence observed at 60X magnification on the sperm bundles was quantified by the NIS-Elements imaging software by choosing 15 random equally sized regions of interest, and the values were compared between *COX4L*-KO and the line of control (w^{1118}). A performed t-test shows statistically significant differences in the observed glowing between these two strains. We interpret this as a reduction in the mitochondria membrane potential and a change in the morphology of the sperm bundles (*COX4L-KO* testes sperm bundles appear to be thinner).

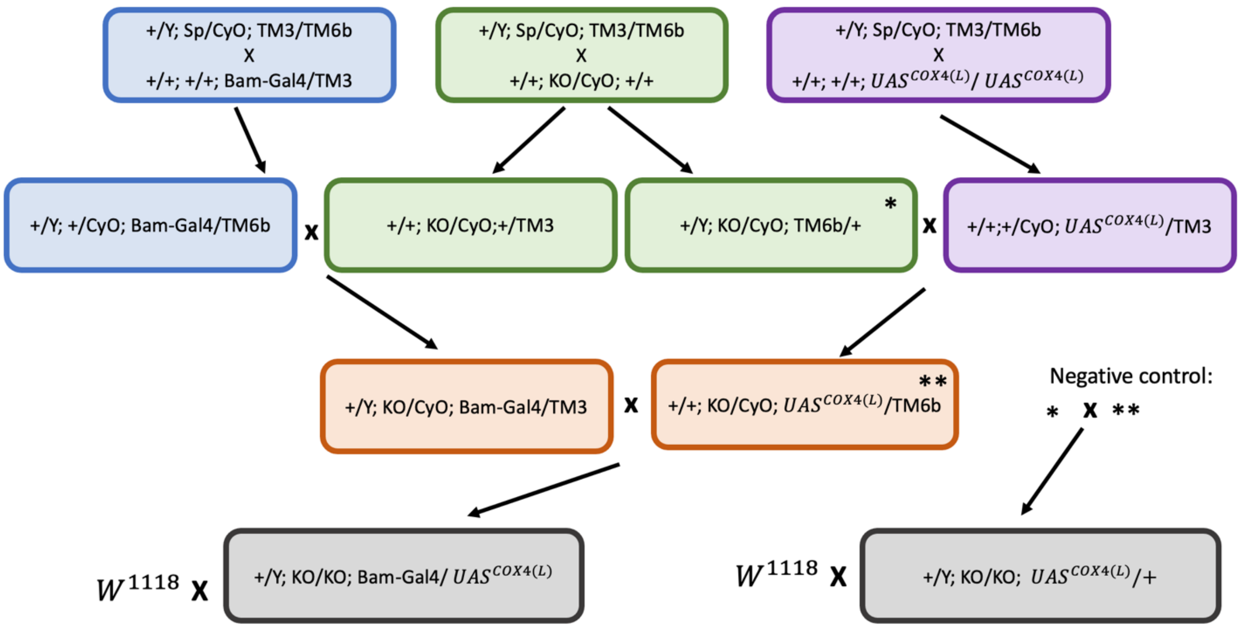
a. COX4 conserved



b. COX4L conserved domains



Supplementary Figure S3. COX4 (a) and COX4L (b) conserved domain analysis.



Supplementary Figure S4. Cross scheme used in this study for the rescue of the *COX4L*-KO phenotype by driving *COX4L*-ORF and *COX4*-ORF with the *bam*- and/or *nos-Gal4* drivers.

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>XP_002090761.1_Drosophila_yakuba_COX4

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