Supporting Online Material for

Manipulating the Metazoan Mitochondrial Genome with Targeted Restriction Enzymes

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Supporting Online Material

Materials and Methods

Molecular biology. The XhoI cDNA was amplified by PCR (5'oligo: `cgggattcatggcattggatctagccgaa`, 3'oligo: `gcctcgagtaacgcggatgcgactct`), fused downstream and in frame with a DNA fragment encoding the N-terminal 40 amino acid residues of *Drosophila* citrate synthase (*CG3861*) and subcloned into the pENTR3C vector. The pMT-*mitoXhoImyc* and pUASp-*mitoXhoImyc* expression constructs were generated by Gateway Cloning system (Invitrogen), using destination vectors from *Drosophila* Gateway collection (T. Murphy lab, Carnegie institute of Washington, Baltimore, MD). The BglII cDNA was amplified (5'oligo: `cgggatccatgaagattgatataacgga`, 3'oligo: `gcctcgagtaatatgtcacgattgttc`) by PCR and pUASp-*mitoBglII* was constructed using the scheme described for *mitoXhoI*.

Stable S2 cell lines expressing pMT-*mitoXhoImyc* were induced with 100 μM $CuSO_4$ for 24 hrs. The total DNA was extracted using Qiamp DNA mini kit (Qiagen #51304) and digested with HindIII alone, or HindIII and XhoI together, fractioned on 1% agrose and transferred onto GeneScreen plus membrane (PerkinElmer #988001). The radioactive probe was made with Prime-It II random primer labeling Kit (Stratagene #300385) using *mt:CoI* cDNA as template. Southern blotting was performed according to the standard protocol (Molecular Cloning, online edition). To genotype mitochondrial DNA, a 4.0 kb DNA fragment flanking the XhoI site (corresponding to mtDNA: 759-4769. 5'oligos:`tggagctattggaggactaaatca`, 3'oligo:`gctcctgttaatggtcatggact`) was amplified by PCR, digested with XhoI, or sequenced (sequencing oligo: `gaattgctcatggtggagcttca`). The majority of mtDNA of each mutant was also amplified by PCR and sequenced. We could not sequence the AT rich region and the 12S rRNA, due to technical difficulty amplifying this part of the genome.

Immunostaining. The S2 cells carrying pMT-*mitoXhoImyc* were induced with 100 μM $CuSO_4$ for 24 hrs, and loaded with 100 nM Mitotracker green (Molecular Probe # M7511) for 30 min. The Cells were fixed with 4% formaldehyde in PBS for 5 min and permeabilized with PBS containing 0.1% Triton X-100. An anti-myc antibody (1:400, Santa Cruz, Sc-789) was used. The images were acquired using a deconvolution microscope (Delta-vision RT; Applied Precision).

Fly genetics. All the flies were maintained on cornmeal-agar-molasses medium at 25 °C under ambient light condition. The transgenic flies expressing MitoXhoI were generated through standard germline transformation procedures. To select for mitochondrial mutations, *UASp-mitoXhoImyc (abbreviatedUASp-mitoXhoI below and in the main text)* flies were crossed to *nanos-GAL4* flies (Bloomington #, 4937). The resulting F1 females (*UASp-mitoXhoI*/+; *nanos-GAL4*/+) were crossed to w^{1118} males. The escaper F2 females were picked and mated with w^{1118} males to establish stocks. mtDNA genotypes were determined by restriction enzyme digestion and sequencing. In all of the experiments, w^{1118} flies were used as a wild type control.

Climbing test. Climbing tests were carried out as previously described [1]. For each test, ten 2-3 day old flies or ten 2-week old flies were transferred to a glass test tube (15 cm long, 1.5 cm diameter) with a mark 10 cm from bottom and parafilm covering the opening. After 2 hr for acclimation, the flies were knocked down to the bottom by gently tapping the tubes. The tubes were held upright under a fiber optic light. The time required for 50% of the flies to climb to the

10 cm line was recorded. Three trials were conducted for each group, and three groups were used for each genotype.

ATP determination. ATP levels were determined as previously described[2]. Five 2-3 day-old male flies of each genotype were homogenized in guanidine extracting buffer (6M Guanidine-HCl, 100mM Tris-HCl, pH7.3). The supernatants were collected after centrifugation. 1/500 dilution was used to determine the ATP level using an ATP determination Kit (Molecular Probes #A22066). The absolute ATP levels were normalized with protein concentration determined by Bradford assay. The average of 3 sets of wild type controls was used as standard for normalization. Each data point is the average of 3 independent experiments.

Cytochrome c oxidase (COX) activity. COX activity was measured as previously described [3] with minor modification. Before being used as a substrate in the assay, commercially purchased cytochrome c (10 mg/ml, equine heart, EMD Biosciences #250600) was reduced with 10 mg/ml ascorbate in sodium phosphate buffer (0.1M sodium phosphate, pH 7.2). The ascorbate was dialyzed out overnight at 4ºC in sodium phosphate buffer using Slide-A-Lyzer Dialysis Cassettes (3500 kDa MW cut-off, Pierce #66330). Reduced cytochrome c was diluted to 25 mM using the extinction coefficient at 550 nm of 29.5 mM^{-1} cm^{-1}. For each experiment, six 2-3 day old male flies were homogenized in 100 µl sodium phosphate buffer containing 0.05% Tween 80, and further diluted by adding 1 mL buffer. Insoluble material was removed by centrifugation at 4000g for 1 minute. A 200 µl aliquot of supernatant was mixed with 750 µl of 25 µM reduced cytochrome c in a plastic cuvette, and the optical density at 550 nm was recorded every 2 sec for 5 min using SWIFT II Wavescan software (version 2.02, Biochrom Ltd). Maximum velocities were calculated by the software and were used to represent relative COX activity. Each bar on the graph is the average of at least 3 independent experiments.

Electron microscopy. To examine retinal morphology, flies were raised under a 12 h light/dark cycle at 25 °C. The hemisected fly heads were fixed in 4% paraformdehyde plus 1% glutaraldehyde in PBS overnight, and post-fixed with 1% Os_2O_4. After dehydration with ethanol series, the samples were embedded in LR white. The 8 nm thin sections were stained with uranyl acetate and lead citrate and viewed on a transmission electron microscopy. The same protocol was also used for TEM analysis of indirect flight muscle, except that the dissected muscle bundles were embedded in 1% low melting agarose after fixation to preserve the tissue integrity. For scanning EM analysis, the flies were dehydrated through ethanol series, sputter-coated and imaged using Hitachi TM-1000 scanning electron microscope.

Sperm motility test. One-week-old male flies were hold away from female flies for 5 days and seminal vesicles were dissected out and gently broken with forceps in Ringer's solution. The bulk of sperm burst out from the seminal vesicles and started to wiggle around. The movement of sperm was videotaped for 15 sec.

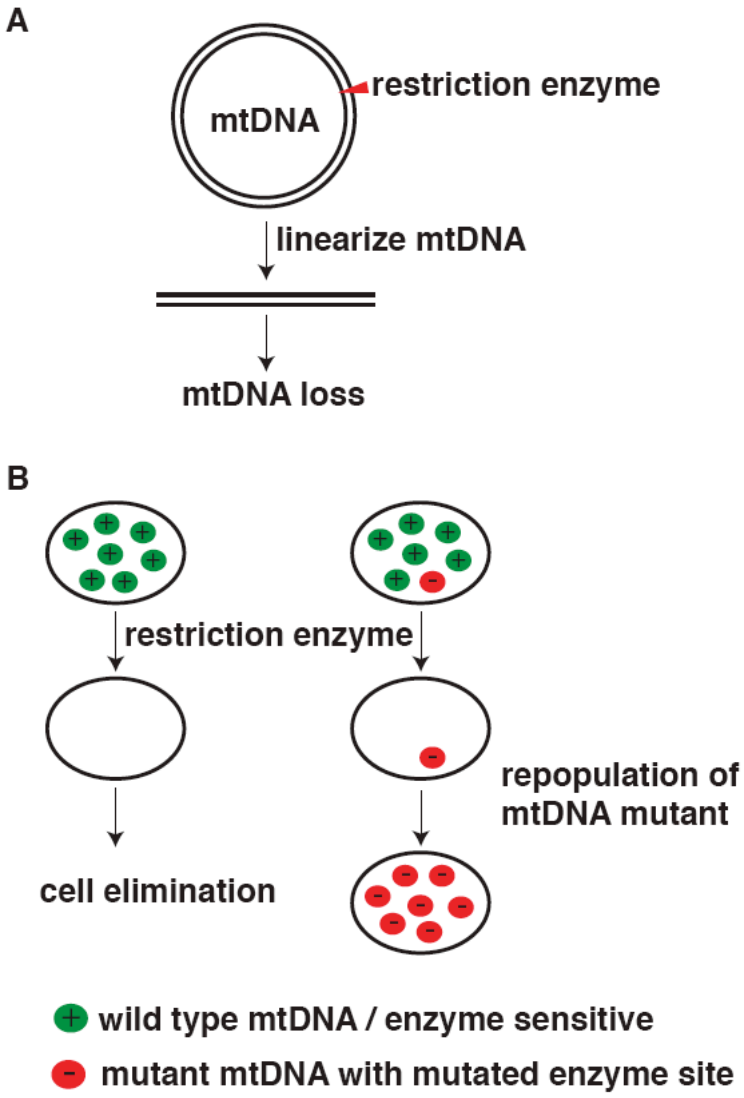

Fig. S1. Hypothesis of mitochondrially targeted restriction enzyme-based selection for mtDNA mutations. (a) Linearization of mtDNA by a restriction enzyme (red arrowhead) will block mtDNA replication, and thereby cause mtDNA loss. (b) In the presence of a mitochondrially-targeted restriction enzyme that can digest wild type mtDNA (green circle), the cells that do not contain any enzyme-resistant mitochondrial genome will lose all of their mtDNA and be eliminated. Those cells harboring enzyme resistant mtDNAs (red circle) will survive the selection by repopulating their mitochondria with these mutant mtDNAs.

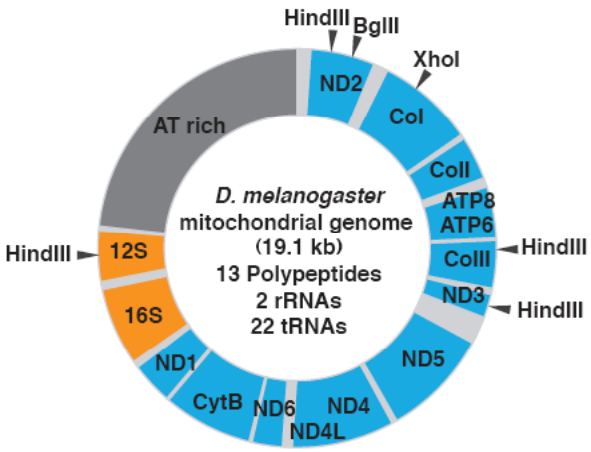

Fig. S2. The mitochondrial genome of *Drosophila melanogaster*. The restriction enzymes used in this study and their relative positions are labeled.

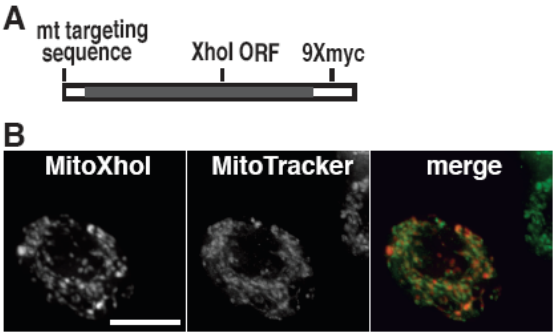

Fig. S4. Targeting restrction enzyme into mitochondria. (A) Schematic view of *mitoXhoI* fusion gene. (B) S2 cells expressing *mitoXhoI* under the control of a metallothionein promoter were induced with 100 μM $CuSO_4$, labeled with MitoTracker (green) and stained with anti-myc antibody (red). Scale bar, 10 μm.

Fig. S3. (A) Alignment of partial amino acid sequences of CoI proteins of *Drosophila* (*Dm*), human (*Hs*), mouse (*Mm*) and *S. cerevisiae* (*Sc*). Conserved amino acid residues are shaded in black. Asterisks label 3 amino acids that may be affected by mutations on XhoI site in *mt:CoI* gene.

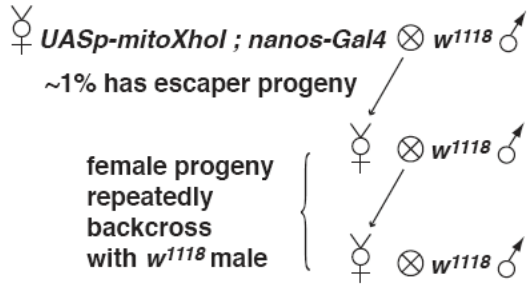

Fig. S5. Cross scheme for selecting *Drosophila* lines carrying XhoI-resistant mitochondrial genomes.

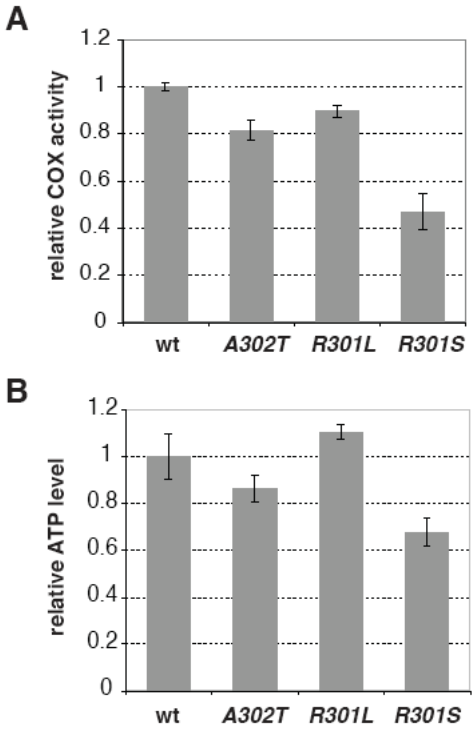

Fig. S6. Biochemical deficiencies of wild type and mtDNA mutants. *mt:CoI*^{R301S} flies showed dramatically reduced COX activity (0.48±0.04 of wild type) and ATP levels (0.67±0.02 of wild type). There were no significant changes in *mt:CoI*^{A302T} and *mt:CoI*^{R301L} flies compared to wild type.

Table S1. Phenotypic comparison of *mt:CoI* mutants in this study with other mutants in electron transport chain complexes in *Drosophila* (A)

	physiological defects							biochemical deficiency	
	viability	**morphology**	**fecundity**	**motility**	**neuropathy**	**myopathy**	**longevity**	**COX activity**	**ATP level**
mt:CoI^{A302T}	viable	normal	normal	normal	no	no	normal	no significant change	no significant change
mt:CoI^{R301L}	viable	normal	male sterile	normal	no	no	normal	no significant change	no significant change
mt:CoI^{R301S} *(B)*	viable	short, thin or missing bristles	male sterile, reduced fertility in female	slow climbing ability	retinal degeneration	age-dependent muscle disorganization	reduced	reduced	reduced
mt-ATP6[4]	viable	normal	NA	"bang"-sensitive	no	muscle degeneration	reduced	normal (C)	reduced
Levy[5]	viable ts- paralysis	NA	NA	NA	CNS degeneration	NA	reduced	reduced	reduced
cyclope (D)[6]	lethal	deformed eye, splitting thorax, smaller bristles	NA	NA	NA	NA	NA	NA	NA
Surf1 (E)[7]	lethal	NA	NA	NA	no CNS-degeneration, defective neurotransmission	abnormal mitochondrial morphology in larval muscle	NA	NA	NA

NA, Not addressed or mentioned in associated studies.

A. The mutants recovered from genetic screening for defective G1-S transitions are not included in this table[8,9,10], as these studies mainly focused on mitochondrial involvement in cell cycle progression, rather than those physiological aspects related to human mitochondrial diseases.
B. *mt:CoI*^{R301S} showed growth retardation, which was absent in the other two *CoI* alleles and not addressed in the studies regarding other mutants in this table.
C. COX activity was not directly determined in the associated study. Normal COX activity is predicted from the result that mutant flies have a normal respiration rate.
D. *cyclope* flies are lethal. The phenotypic characterizations are either based on heterozygote flies or mitotic clones.
E. There is no classical mutant available for *Surf1* gene. The study was conducted using RNAi knockdown. Ubiquitous knockdown of *Surf1* results in lethality. Some phenotypic assessments were based on tissue specific RNAi.

Table S2. The restriction enzymes with one site in the *Drosophila* mitochondrial genome

Enzyme	Position	Sequence	Locus (location)
BmrI	193	ACTGGG	*mt:tRNA:F* (171-239)
BsmFI *	288	GGGACnnnnnnnnnnnnnn	*mt:ND2* (240-1265)
BsrBI	599	CCGCTC	*mt:ND2*
BglII	801	AGATCT	*mt:ND2*
EcoRV	1360	GATATC	*mt:tRNA:C* (1322-1383)
NruI	1474	TCGCGA	*mt:CoI* (1470-3009)
BsgI	1627	GTGCAGnnnnnnnnnnnnnnn	*mt:CoI*
BstZ17I	2006	GTATAC	*mt:CoI*
Tsp45I *	2183	GTsAC	*mt:CoI*
XhoI	2369	CTCGAG	*mt:CoI*
BbvCI	2384	CCTCAGC	*mt:CoI*
EciI	2950	GGCGGAnnnnnnnnnnn	*mt:CoI*
NsiI	3159	ATGCAT	*mt:CoII* (3083-3767)
SapI	3311	GCTCTTCnnnn	*mt:CoII*
NciI	3647	CCsGG	*mt:CoII*
DrdI	4246	GACnnnnnnGTC	*mt:ATP6* (4062-4736)
BssSI	4923	CACGAG	*mt:CoIII* (4736-5524)
StyI	4939	CCwwGG	*mt:CoIII*
PleI	5301	GAGTCnnnnn	*mt:CoIII*
AhdI	5463	GACnnnnnGTC	*mt:CoIII*
HpaI	6752	GTTAAC	*mt:ND5* (6401-8124)
AflII	7418	CTTAAG	*mt:ND5*
PstI	7515	CTGCAG	*mt:ND5*
Bsu361	9614	CCTnAGG	*mt:ND4L* (9544-9834)
BsaBI *	10672	GATnnnnATC	*mt:CytB* (10498-11634)
BsmBI *	10707	CGTCTCnnnnn	*mt:CytB*
BciVI	10882	GTATCCnnnnnn	*mt:CytB*
NdeI	11657	CATATG	*mt:tRNA:S* (11637-11702)
BbsI	13155	GAAGACnnnnnn	*mt:lRNA* (12734-14058)
BsrGI	14208	TGTACA	*mt:sRNA* (14131-14916)
BanI	14742	GGyrCC	*mt:sRNA*

n = A or C or G or T r = A or G s = C or G w = A or T y = C or T

There total 31 single enzyme sites excluding the isochizomers. The *Drosophila melanogaster* full mitochondrial DNA sequence (# U37541) was used to determine the restriction sites.
*These four enzymes require high temperature (50°C-65°C) for optimal activity.

References

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Supplemental Movies

Movie S1. Wild type sperm.
Movie S2. Sperm of *mt:CoI*^{R301L} fly.