File S1

Supporting Materials And Methods

Testing for the presence of *Wolbachia* by PCR

Following the previously published procedure (O'NEILL *et al*. 1992), insect ovary tissue was dissected, homogenized and incubated with 2 µl of proteinase K (10 mg/ml) for 30min at 37°C, followed by 5 min at 95°C. Samples were briefly centrifuged, and 1 µl of the supernatant was used as the template in subsequent PCR, with *Wolbachia* 16S rRNA-specific primers or universal bacterial 16S rRNA primers (see Table S2), with 30 cycles of 95 °C, 1 min, 52 °C 1 min, 72 °C 1 min.

SDS-PAGE and Western blotting

Gels were run in a Mini-Protean® II electrophoresis cell (Bio-Rad) then transferred to nitrocellulose membranes (Invitrogen) membranes using the iBlot™ dry blotting system (Invitrogen). After blotting, membranes were blocked in 5% non-fat milk in TBST buffer (150 mM M NaCl, 50 mM Tris-HCl, 0.05% Tween 20, pH 7.6) for 1 h at room temperature, then incubated with the primary antibody in 1% non-fat milk in TBST buffer overnight at 4 ° C. After washing 4 x 15 min in TBST buffer and blocking again in the blocking buffer for 1 h at room temperature, membranes were incubated in the presence of the secondary antibody for 1 h at room temperature, followed by further washes (4 x 15 min) in TBST buffer. Detection was performed with the Amersham ECL™ Western blotting analysis system (GE Healthcare).

Preparation of mitochondria for mtDNA isolation

Batches of 50-200 flies were crushed with one stroke of a Teflon pestle in a glass homogenizer in 4 ml HB medium (225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 0.1% BSA, 10 mM Tris-HCl pH7.6) then homogenized with 8 more strokes. The homogenate was transferred to a 15 ml plastic centrifuge tube, filled up with HB, then centrifuged for 5 min at 1,000 g_{max} at 4 °C to pellet cell debris and nuclei. The supernatant was decanted to a new 15 ml tube and crude mitochondria were pelleted for 10 min at 12,000 g_{max} at 4 °C. The pellet was washed once, then resuspended in 300 µl HB and overlaid onto a 1.5 M/1 M sucrose (250 + 250 µl) step gradient and centrifuged for 1 h at 45,000 g_{max} at 4 °C. The mitochondrial layer was transferred to a 2 ml tube and one volume of HB was added. Mitochondria were pelleted for 5 min at 12,000 g_{max} at 4 °C, then processed immediately for DNA extraction.

Repeat analysis of the NCR by end-labeling and partial restriction digestion

Primers Dm14428F and Dm225R (see Table S2), designed to amplify the entire NCR, were individually end-labeled using T4 polynucleotide kinase (T4 PNK, Fermentas), and [γ-^{32}P]ATP (Perkin Elmer, 6000 Ci/mmol) under manufacturer's recommended conditions. Each 20 µl reaction contained, in manufacturer's Reaction Buffer A, 1 nmol of primer, 10 u of

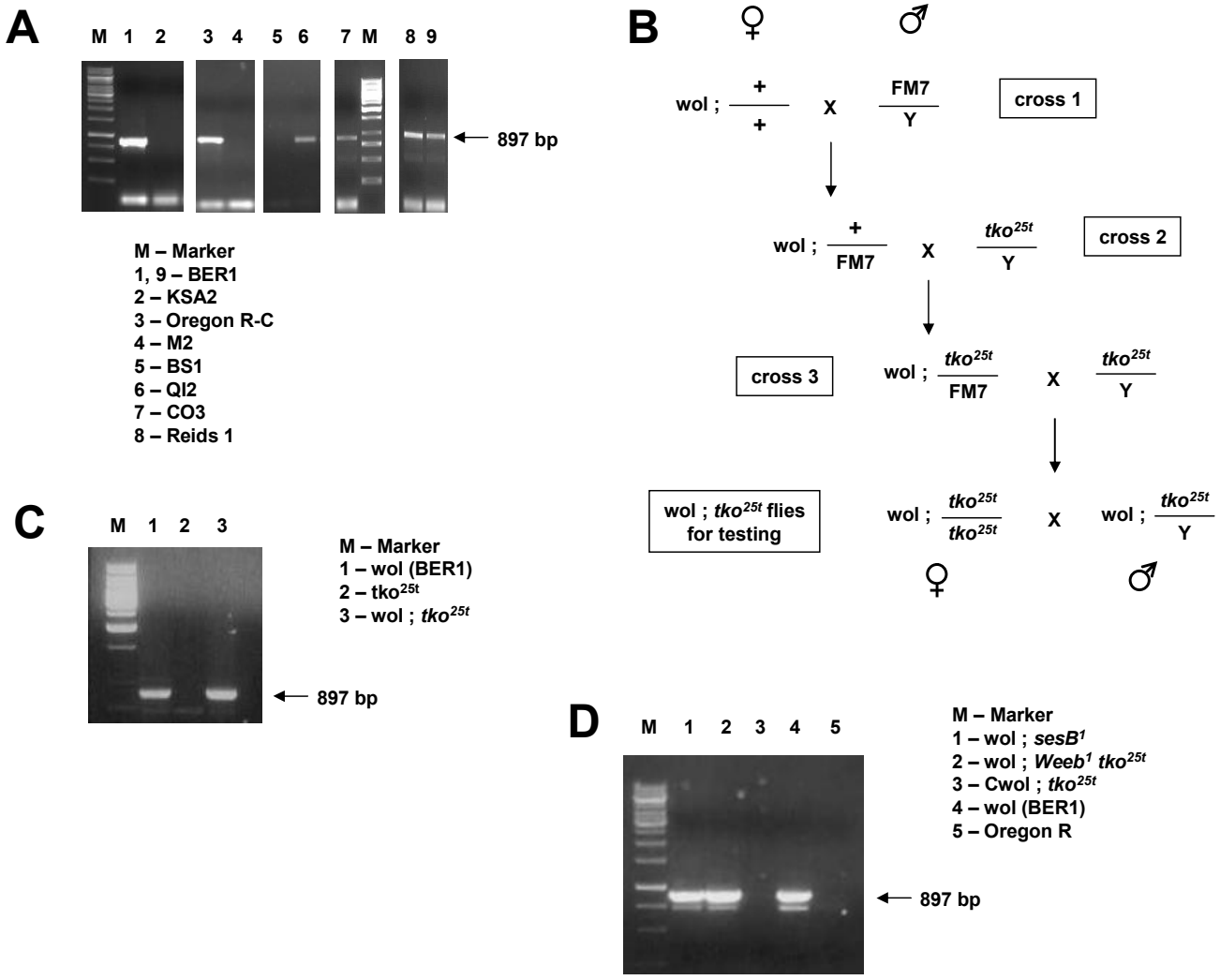
enzyme and 3 µl (30 µCi) of labeled ATP. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1 µl of 0.5 M EDTA (pH 8.0) and further incubation at 75 °C for 10 min. The unincorporated label was removed by gel filtration on Sephadex G-50 (GE healthcare). The mtDNA noncoding region was amplified from partially purified mtDNA with end-labeled primer pairs (for 'left' end: ^{32}P-Dm14428F and Dm225R, for 'right' end: Dm14428F and ^{32}P-Dm225R) using LongRange PCR kit (Qiagen), under the following conditions: 93 °C, 3 min (initial denaturation), followed by 35 cycles of 93 °C, 15 s (denaturation); 50 °C, 30 s (annealing); 60 °C, 6 min (extension), with final extension at 60 °C for 10 min. Restriction site mapping by partial digestion and gel electrophoresis was performed essentially as described previously (LEWIS *et al*. 1994). Briefly, after gel purification (GeneJet gel extraction kit, Fermentas), the radiolabeled fragments (20 ng in 20 µl) were partially digested with *Pac*I (1 unit, 1 x NEBuffer 1, Biolabs), *Hpy*188I (0.6 unit, 1 x NEBuffer 4, Biolabs) or *Swa*I (1 unit, 1 x buffer O, Fermentas). Reactions were incubated for 6 min (*Pac*I and *Hpy*188I at 37 °C, *Swa*I reaction at 30 °C), then terminated by the addition of EDTA to 20 mM. The reactions and 1 kb labeled DNA marker (GeneRuler^{TM} 1 kb ladder, 250-10,000 bp, Fermentas, labeled by [γ-^{32}P]ATP as above described above) were fractionated by 1.2% agarose gel electrophoresis in TAE buffer, after which the gel was dried (model 583, BIO-RAD) and exposed overnight at -80 °C (FUJI medical X-RAY film), with intensifying screen.

Long PCR and mtDNA sequencing

The coding region of the mitochondrial genome was amplified in three overlapping fragments (each about 5.5 kb in size) by long-PCR (LongRange PCR kit, Qiagen), using partially purified mtDNA as template. The cycling conditions were: 93 °C, 3 min (initial denaturation); then 35 cycles of 93 °C, 15 s (denaturation); 50 °C, 30 s (annealing); 60 °C, 6 min (extension), with final extension at 60 °C for 10 min. Each PCR product was verified by 0.8% (w/v) agarose gel electrophoresis, then purified over mini-spin columns and used as templates for cycle sequencing using primers as indicated (Table S2). The mtDNA fragments were sequenced in both directions using Big Dye v3.1 chemistry and a 3130xl genetic analyzer (Applied Biosystems), with primer sequences as listed in Table S2. The sequences were aligned manually in win_serialcloner1-3 (Serial Basics), then analyzed using the phred/phrap/consed package (GORDON *et al*. 1998). Each deviation from the reference sequence was confirmed by a second PCR and sequencing reaction. The NCR and its flanking sequences were sequenced separately, including the portion of the SSU rRNA not already covered by sequencing of coding region fragment 3.

For sequencing of the NCR, repeat arrays I and II, plus their flanking regions, were amplified from mtDNA template in separate, overlapping PCR reactions, then analyzed by a combination of cloning of restriction fragments and sequencing with overlapping primers, as indicated below. In general, at least three independent PCR reactions and over 200 clones were analyzed to infer the sequence of each sub-region from each strain analyzed. Nucleotide differences found only in single clones were assumed to represent mutations arising during PCR/cloning, but might also represent low-level

heteroplasmy. PCR used an initial denaturation step at 93 °C for 3 min, followed by 35 cycles of 93 °C for 15 s, 42 °C for 30 sec, 60 °C for 1-3 min depending on the length of the desired product, with a final extension at 60 °C for 10 min. PCR products were purified by gel electrophoresis (GeneJet gel extraction kit, Fermentas). The 'left' end of the NCR, between SSU rRNA and repeat array I, plus the short region of the SSU rRNA gene not already covered by sequencing of coding region fragment 3, was inferred by direct sequencing of the product amplified with primer pair Dm14428F/Dm17556R, using overlapping primers Dm14428F, Dm14570F, Dm14721F and Dm14787R. The PCR for type I repeats, which also confirmed the reverse-strand portion of the flanking region and start of the SSU rRNA gene, was performed using primer pair (Dm14721F and Dm17556R). After gel purification, the product was digested with *Ssp*I (1 X buffer G, Ferments) or *Pac*I at 37 °C overnight, then ligated into plasmid vector pNEB193 (New England Biolabs), which had been digested, respectively, with *Sml*I (*Pme*I, Fermentas) at 30 °C or *Pac*I (New England Biolabs) at 37 °C, then dephosphorylated with CIAP (Fermentas) and purified by gel electrophoresis. The order of repeat I elements was inferred from overlaps of the restriction sites used for cloning (see Fig. S4F). It was further confirmed by PCR using primer pair Dm15578F/Dm17295R, and an elongation time of 90 s, which generated a mixture of 3 fragments of approximately 330, 700 and 1100 bp, which were independently cloned and sequenced. The region between repeat array I and repeat array II was amplified using PCR primer pairs Dm15578F/Dm17556R and Dm15578F/Dm17833R, and sequenced using unique sequencing primers covering this region on both strands (Table S2). Repeat array II was amplified by four different primer pairs which, under varying extension times, generate products of different sizes (see Fig. S4G). These were independently cloned and sequenced, to infer the final order of repeat II elements, which are very similar. The primer pairs used for repeat array II were: Dm15578F/Dm17833R, Dm17556F/Dm18026R, Dm17717F/Dm17833R, and Dm18933F/Dm225R. PCR products were cloned into pCR4 vector (TA Cloning Kit, Invitrogen) after gel purification, and sequenced using universal M13 forward and reverse primers. The shortest product from primer pair Dm15578F/Dm17833R allowed unambiguous assignment of the first portion of the ‘first’ copy of repeat II (the one closest to the repeat I array). The next shortest product from this primer pair extends into the second copy of repeat II, allowing full assignment of the first copy and the junction with the second. Similarly, the two shortest products from primer pair Dm18933F/Dm225R allow unambiguous assignment of the final copy of repeat II (the one closest to the $tRNA^{Ile}$ gene), as well as the ‘right’ end of the NCR between repeat array II and $tRNA^{Ile}$, extending into the coding region. The various products from the two other primer pairs allow unambiguous assignment of the sequence and order of the remaining repeat II elements. All sequences were assembled using win_serialcloner1-3.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897 bp
M – Marker
1, 9 – BER1
2 – KSA2
3 – Oregon R-C
4 – M2
5 – BS1
6 – QI2
7 – CO3
8 – Reids 1
B
♀
♂
wol ; + / + X FM7 / Y
cross 1
wol ; + / FM7 X tko25t / Y
cross 2
cross 3
wol ; tko25t / FM7 X tko25t / Y
wol ; tko25t flies for testing
wol ; tko25t / tko25t X wol ; tko25t / Y
C
M 1 2 3
897 bp
M – Marker
1 – wol (BER1)
2 – tko25t
3 – wol ; tko25t
D
M 1 2 3 4 5
897 bp
M – Marker
1 – wol ; sesB1
2 – wol ; Weeb1 tko25t
3 – Cwol ; tko25t
4 – wol (BER1)
5 – Oregon R

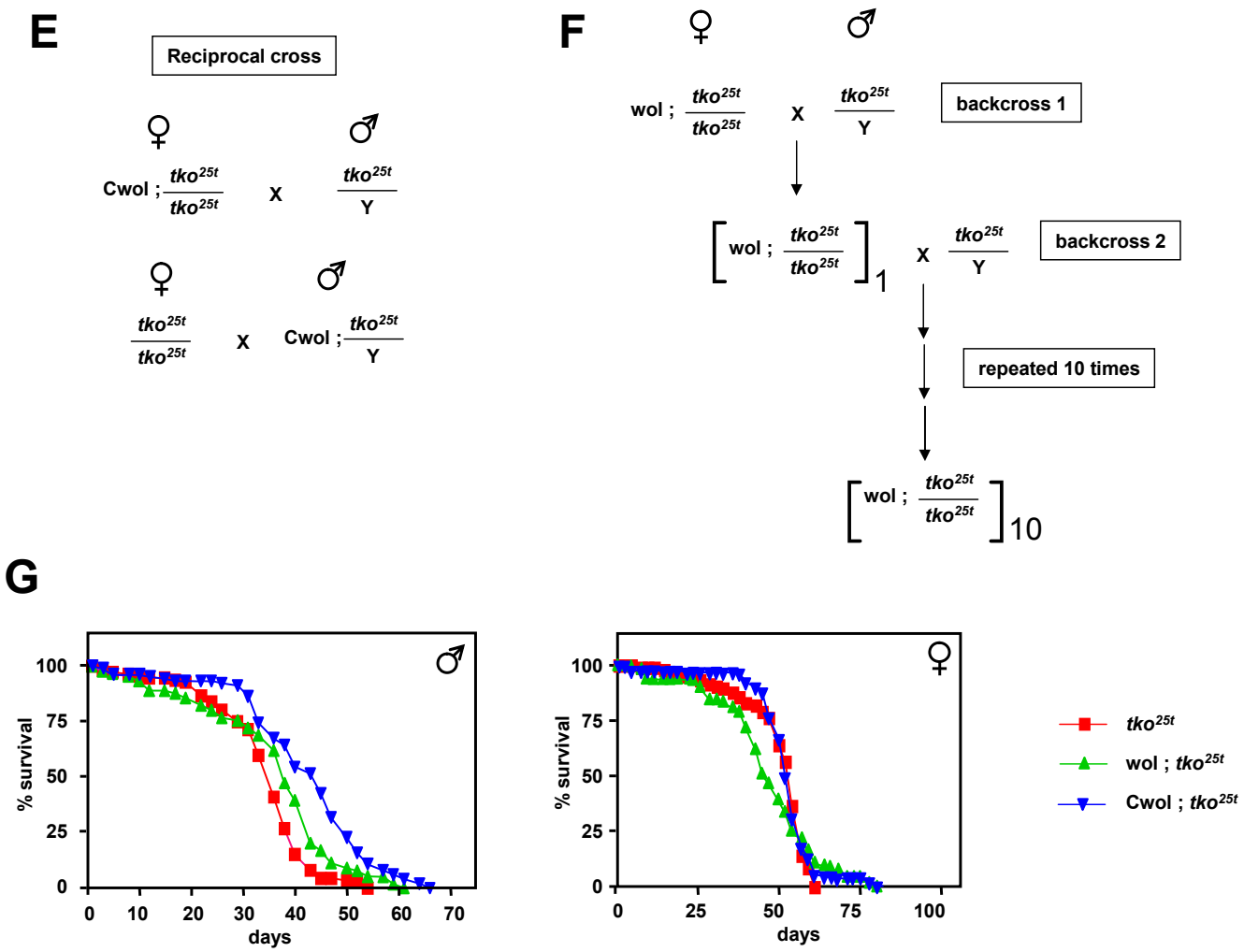
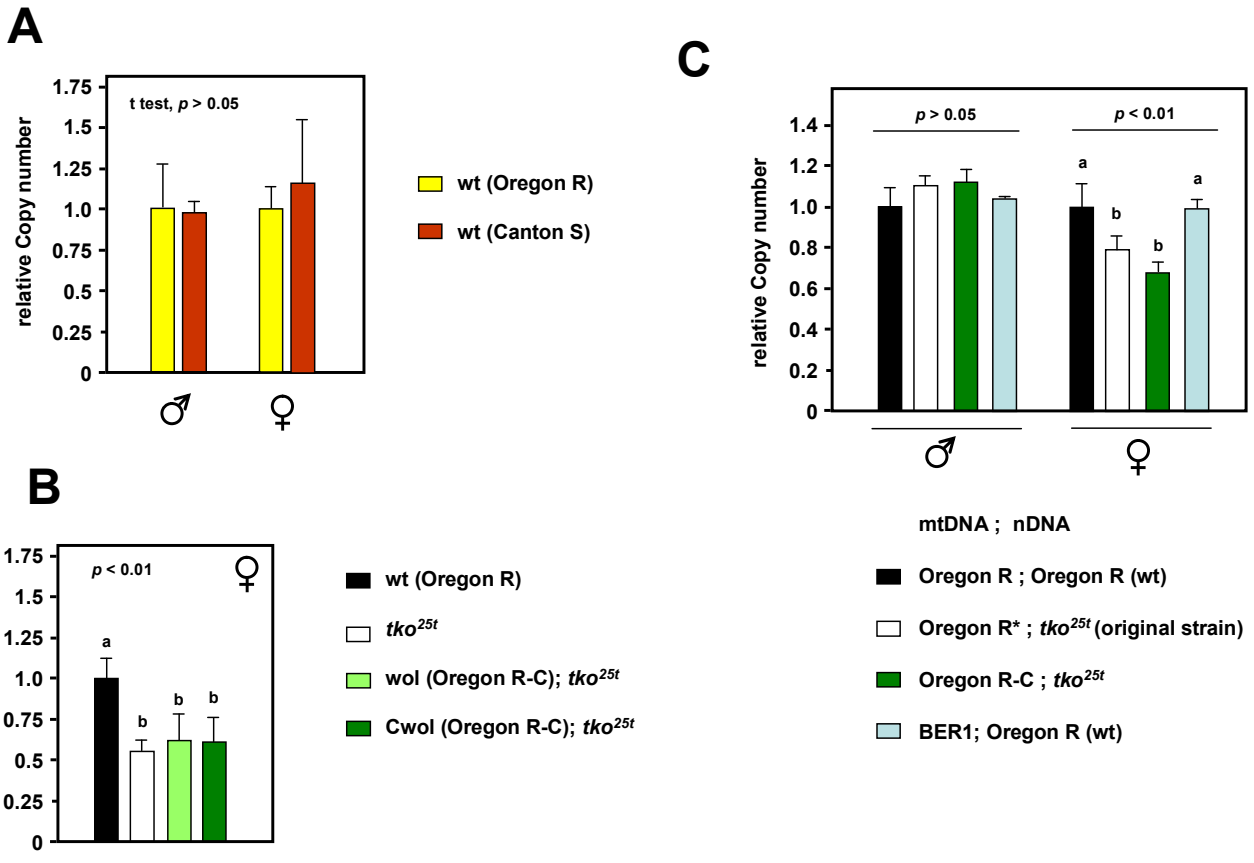


Figure S1 Creation and phenotypic characterization of cybrid *tko*^{25t} and *sesB*^{1} flies. (A) Representative gels of PCR products obtained using the *Wolbachia* 16S rRNA gene primers on genomic DNA of single individuals from the wild-type strains indicated. Gels run in parallel using the same marker ladder. Multiple individuals from these strains gave consistent findings. Infected strains BER1, Oregon R-C, QI2 and CO3 were maintained in quarantine and females were crossed into the *tko*^{25t} background using the crossing scheme shown in (B). Experimental crosses to test the effects of *Wolbachia* cytoplasms on the *tko*^{25t} phenotype used the *Wolbachia*-infected progeny from cross 3 (i.e. lacking the FM7 balancer), which were also maintained subsequently as both balanced and homozyous stocks. The presence of *Wolbachia* cytoplasm is denoted by wol. The use of the X-chromosome balancer excludes any suppressor effects that may be linked to *tko* itself, such as the segmental duplication studied previously (KEMPPAINEN et al. 2009). (C) The presence of *Wolbachia* from strain BER1 after introgressing its cytoplasm into the *tko*^{25t} nuclear background was confirmed by PCR, with template genomic DNAs as indicated. (D) *Wolbachia* was introduced into the *sesB*^{1} background by an identical strategy, and its presence confirmed by PCR, as shown. Its removal from the infected *tko*^{25t} line by tetracycline treatment (Cwol) was confirmed similarly, and by PCR with universal bacterial 16S rRNA gene primers (Table S2) which also gave no product. wol ; *Weeb*^{1} *tko*^{25t} – *Wolbachia*-infected *Weeble*^{1} *tko*^{25t} suppressor strain (KEMPPAINEN *et al*. 2009). (E) Reciprocal crossing scheme to test inheritance pattern of the suppressor phenotype of the BER1-derived *Wolbachia*-free Cwol ; *tko*^{25t} line. The demonstration of strictly maternal inheritance of the suppressor (Fig. 1D) rules out any significant contribution from nuclear DNA. (F) Backcrossing scheme to confirm the cytoplasmic inheritance of *tko*^{25t} suppression in the *Wolbachia*-infected strain BER1 (denoted wol). Note that this back-crossing scheme was also used to test other cytoplasms (Fig. 4), producing identical results for four different suppressors, again consistent with the suppressor determinant being purely cytoplasmic. (G) Lifespan curves for flies of the genotypes indicated. The curves for *Wolbachia*-infected (wol) and cured (Cwol) *tko*^{25t} males in the presence of BER1 mtDNA, after backcrossing for 10 generations, were significantly different (median survival of 43 d) from *tko*^{25t} males in the original (Oregon R-related) mtDNA background (median lifespan 31 d), $p < 0.0001$, log rank test). Because the flies are backcrossed to the same nuclear background, the differences are strictly attributable to the cytoplasmic genotype. Note that *tko*^{25t} flies have a much shorter lifespan than wild-type Oregon R flies tested in our laboratory (SANZ *et al*. 2010a).


A
t test, p > 0.05
relative Copy number
1.75
1.5
1.25
1.0
0.75
0.5
0.25
0
♂
♀
wt (Oregon R)
wt (Canton S)
B
p < 0.01
♀
relative Copy number
a
b
b
b
wt (Oregon R)
tko25t
wol (Oregon R-C); tko25t
Cwol (Oregon R-C); tko25t
C
p > 0.05
p < 0.01
relative Copy number
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
a
b
b
a
♂
♀
mtDNA ; nDNA
Oregon R ; Oregon R (wt)
Oregon R* ; tko25t (original strain)
Oregon R-C ; tko25t
BER1; Oregon R (wt)

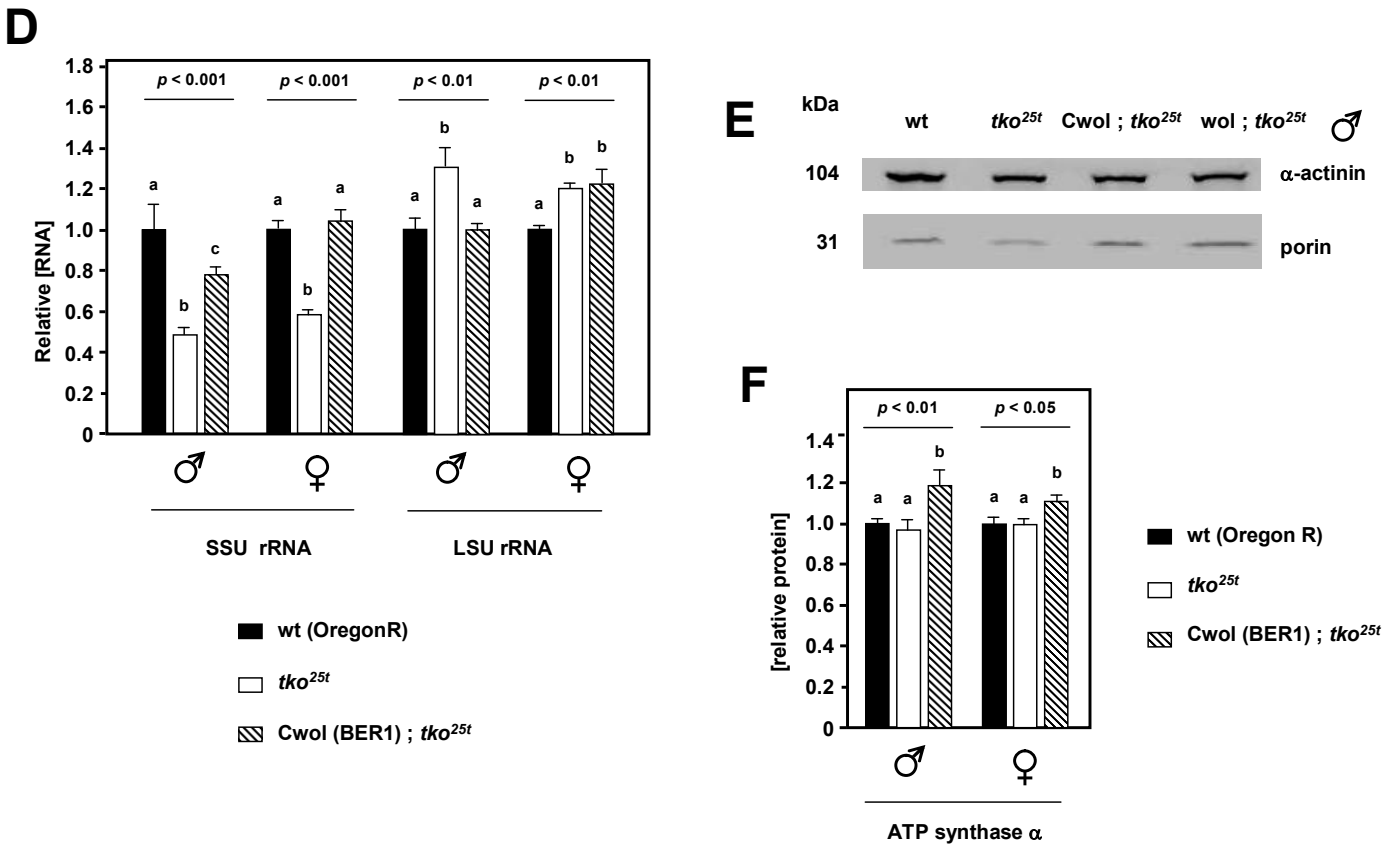


Figure S2 Molecular correlates of partial suppression of *tko*^{25t} by the suppressor mtDNA backgrounds. (A) Supplementary data to Fig. 2D: copy number of mtDNA in two different wild-type strains is similar. Copy number, relative to 18S rDNA, means ± SD, was normalized to that in Oregon R flies of the same sex. The copy number differences seen in *tko*^{25t} flies in different backgrounds (Fig. 2D) are outside the range of variation due to strain background only. (B) Supplementary data to Fig. 2D: the copy number of mtDNA in female *tko*^{25t} flies is significantly lower than in wild-type flies also in a second non-suppressor mtDNA background (Oregon R-C). Data from first two columns reproduced from Fig.2D (experiments were carried out in parallel). (C) Further supplementary data to Fig. 2D: mtDNA copy number is not altered by BER1 mtDNA in a wild-type nuclear background. a, b denote significantly different data classes. Oregon R* denotes the mtDNA of the original *tko*^{25t} strain, whose sequence is very similar (but not identical) to Oregon R or Oregon R-C (Table S1). (D) Supplementary data to Fig. 2E: levels of mitoribosomal SSU and LSU RNA levels in flies of the indicated genotypes relative to the *mRpL32* mRNA standard, means ± SD, normalized to the values in wild-type Oregon R flies of the given sex. The same data were used to compute the SSU/LSU ratios plotted in Fig. 2E. The decreased ratio of SSU to LSU rRNA in *tko*^{25t} flies consists of a decrease in SSU rRNA combined with a small, perhaps compensatory increase in that of LSU rRNA, as reported previously (KEMPPAINEN *et al*. 2009). (E) Representative Western blot of protein extracts from males flies of the indicated genotype, probed for porin (mitochondrial outer membrane marker) and for α-actinin (cytosolic loading control). The global amount of mitochondria, as measured by this assay, is decreased in the presence of the *tko*^{25t} mutation in the original (Oregon R-related) mtDNA background, but restored to wild-type levels in the BER1 background, whether *Wolbachia* are present (wol) or absent (Cwol). The summary data of Fig. 2F are compiled from densitometry of this and equivalent blots. (F) Levels of a representative subunit of complex V (ATP synthase, subunit α), based on densitometry of Western blots, normalized against a cytosolic loading control (α-actinin). In all panels a and b denote significantly different data classes (Newman-Keuls test, $p < 0.05$, based on ANOVA, p values as indicated, each sex and, where appropriate, each gene considered separately).

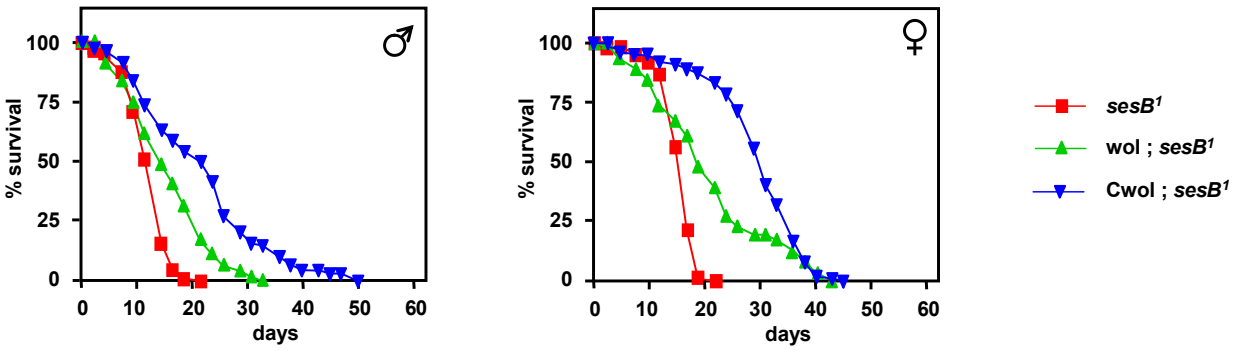
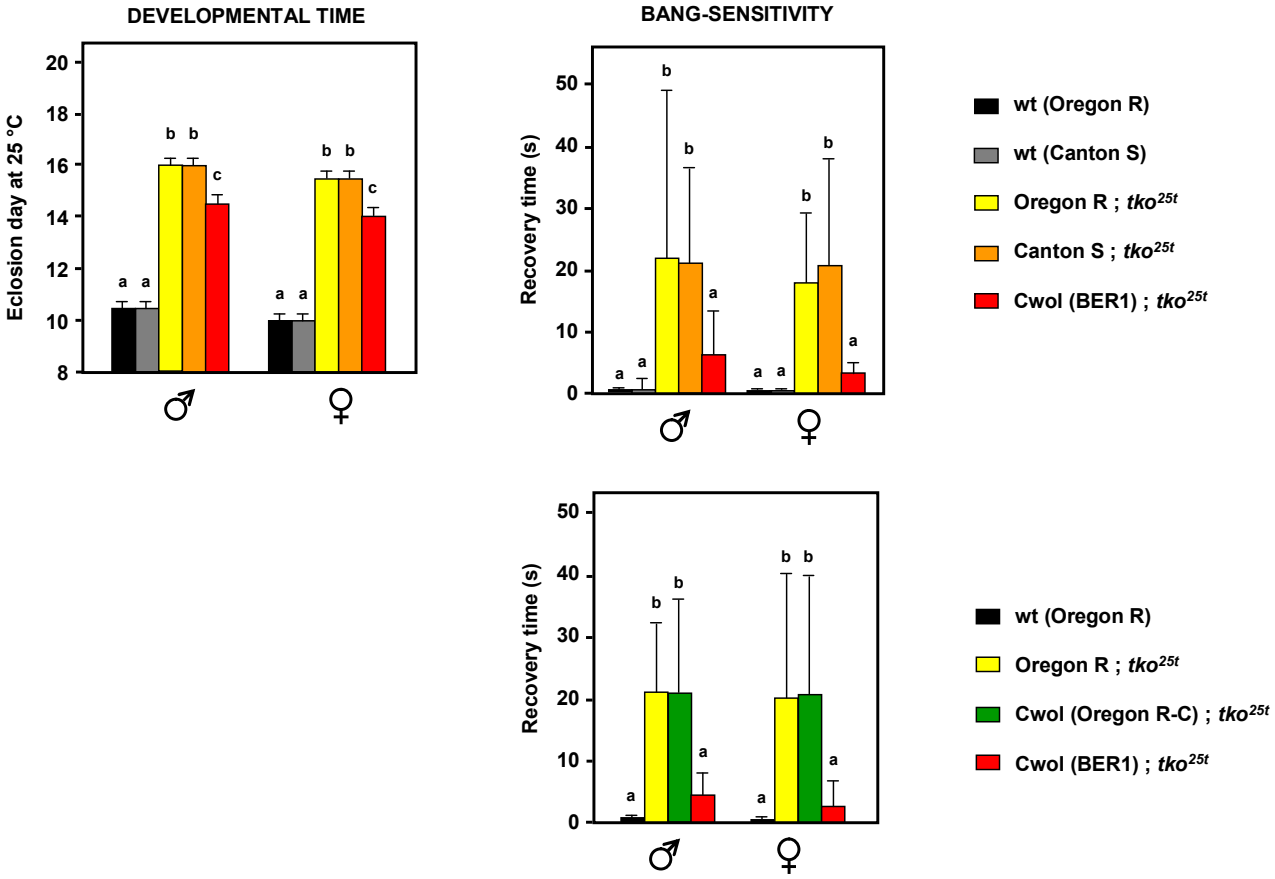
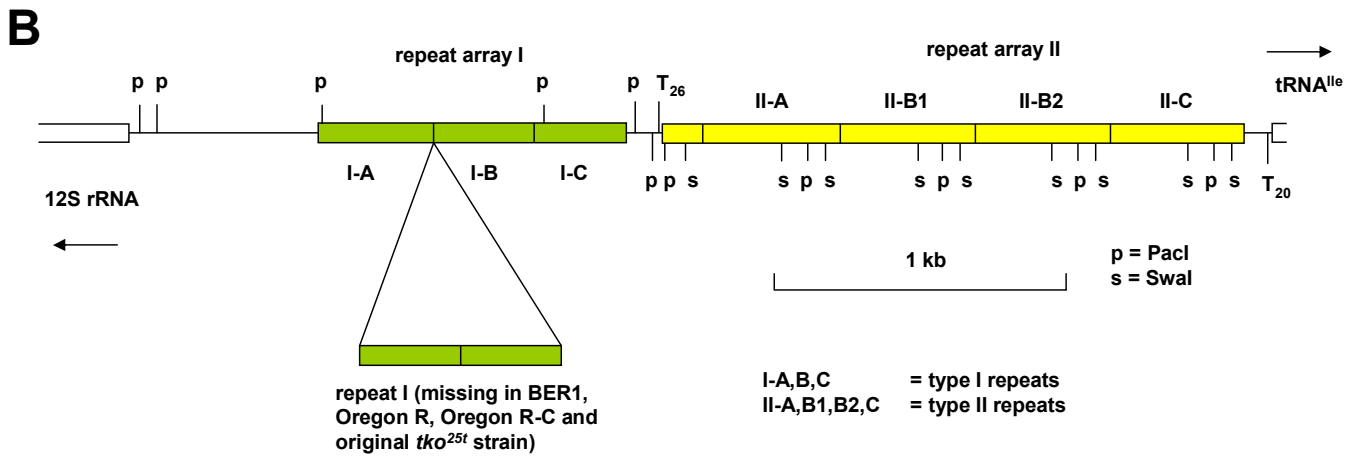
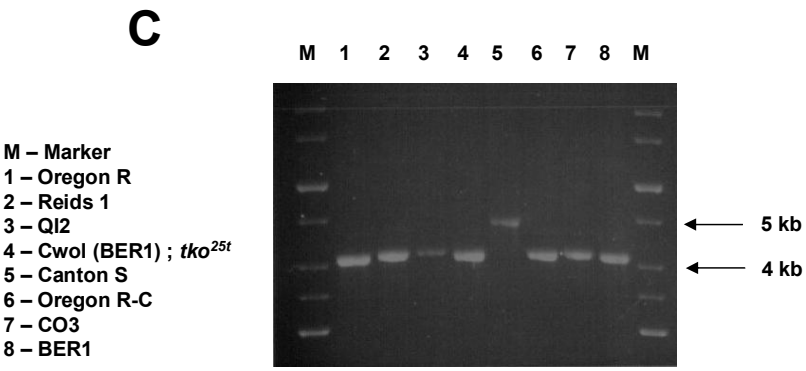


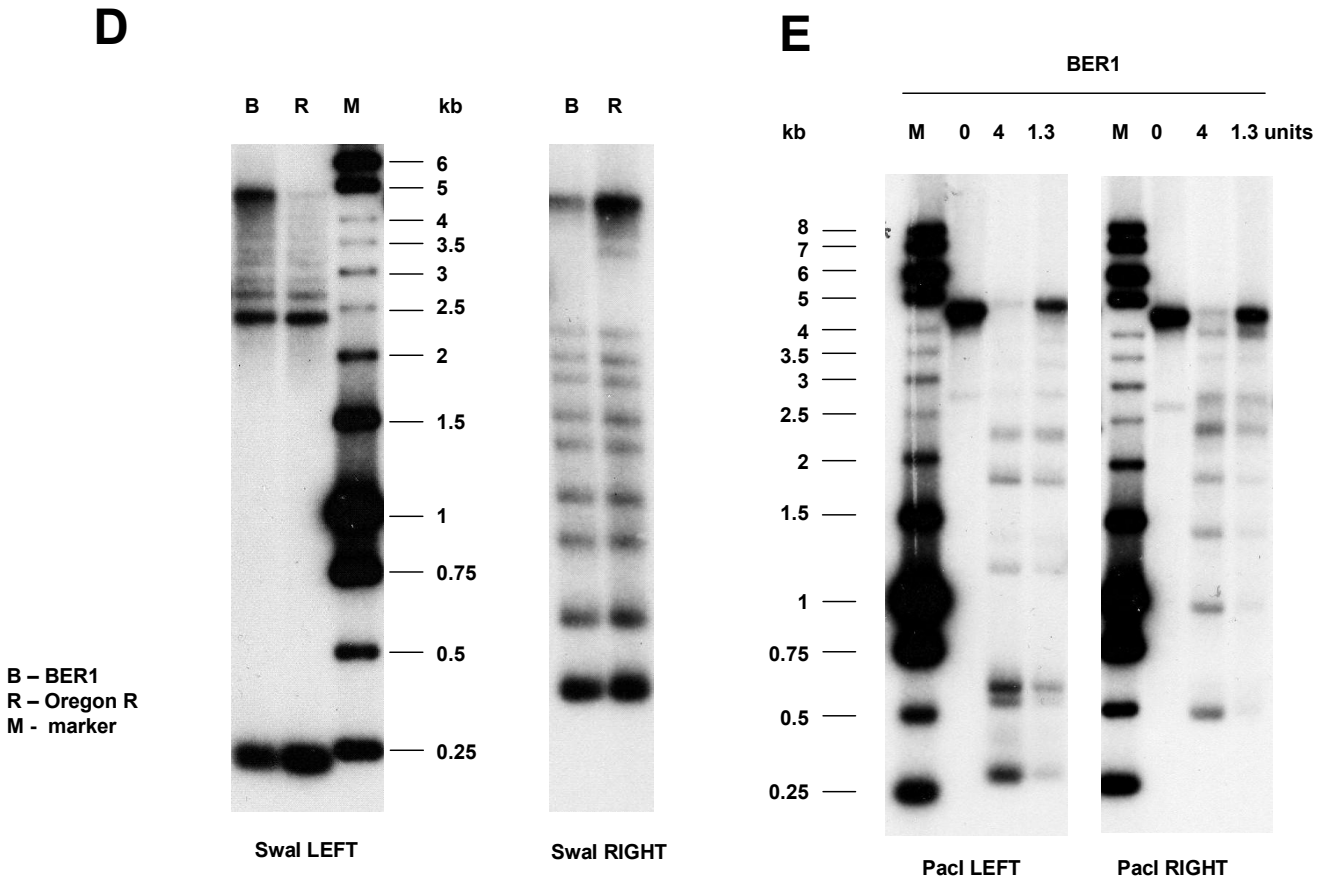
Figure S3 BER1 strain mtDNA increases the lifespan of *sesB*^{1} flies. Lifespan curves for flies of the genotypes indicated. For both sexes, the curves for wol (BER1); *sesB*^{1} and Cwol (BER1) ; *sesB*^{1} (median survival of 17, 19 d, respectively, for males, 31 and 26 d for females) were significantly different from *sesB*^{1} flies of the same sex (median survival 10 d for males, 15 d for females, $p < 0.0001$, log rank test, in all cases). Since the flies are backcrossed to the same nuclear background, the differences are strictly attributable to the cytoplasmic genotype. BER1 mtDNA thus mitigates the age-related degeneration of *sesB*^{1} adults, even though it does not modify the *sesB*^{1} developmental phenotype.

A

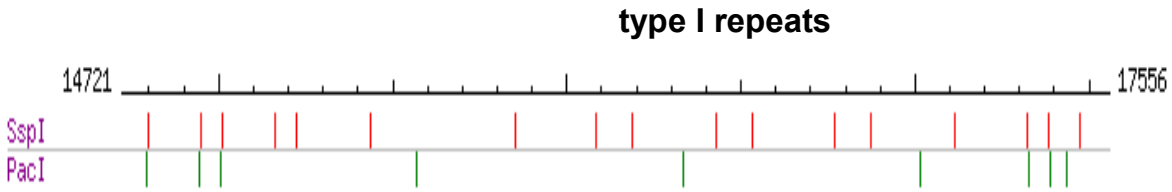

DEVELOPMENTAL TIME
Eclosion day at 25 °C
20
18
16
14
12
10
8
a a
b b
c
a a
b b
c
♂
♀
BANG-SENSITIVITY
Recovery time (s)
50
40
30
20
10
0
a a
b
b
a
a a
b
b
a
♂
♀
wt (Oregon R)
wt (Canton S)
Oregon R ; tko25t
Canton S ; tko25t
Cwol (BER1) ; tko25t
Recovery time (s)
50
40
30
20
10
0
a
b
b
a
a
b b
b b
a
♂
♀
wt (Oregon R)
Oregon R ; tko25t
Cwol (Oregon R-C) ; tko25t
Cwol (BER1) ; tko25t


B
repeat array I
repeat array II
p p
p
p
p
T26
tRNAIle
II-A
II-B1
II-B2
II-C
I-A
I-B
I-C
12S rRNA
p p s
s p s
s p s
s p s
s p s
T20
1 kb
p = PacI
s = SwaI
repeat I (missing in BER1, Oregon R, Oregon R-C and original tko25t strain)
I-A,B,C = type I repeats
II-A,B1,B2,C = type II repeats


C
M 1 2 3 4 5 6 7 8 M
M – Marker
1 – Oregon R
2 – Reids 1
3 – QI2
4 – Cwol (BER1) ; tko25t
5 – Canton S
6 – Oregon R-C
7 – CO3
8 – BER1
5 kb
4 kb


D
B R M kb
6
5
4
3.5
3
2.5
2
1.5
1
0.75
0.5
0.25
B – BER1
R – Oregon R
M - marker
SwaI LEFT
B R
SwaI RIGHT
E
BER1
kb
M 0 4 1.3
M 0 4 1.3 units
8
7
6
5
4
3.5
3
2.5
2
1.5
1
0.75
0.5
0.25
PacI LEFT
PacI RIGHT

F


type I repeats
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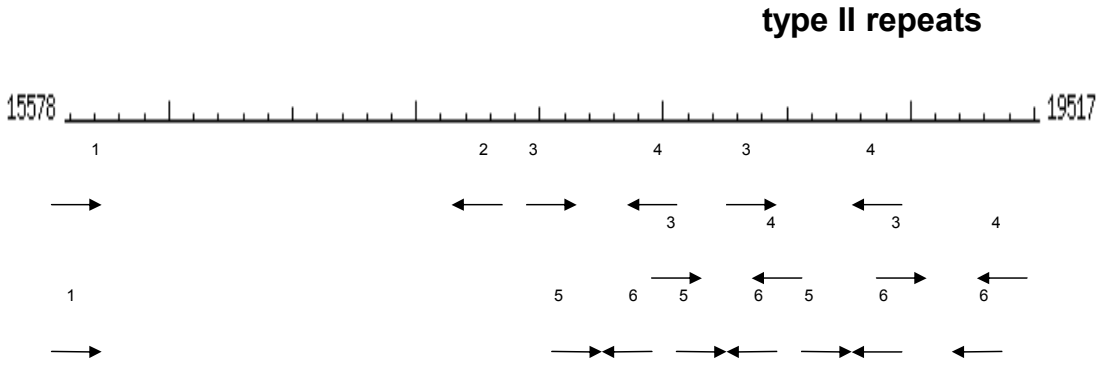
17556
SspI
PacI

G


type II repeats
15578
19517
1
2
3
4
3
4
3
4
3
4
1
5
6
5
6
5
6
6

H

Black = Refseq (NCBI accession NC_001709)
Blue = tko25t (original mtDNA background, NCBI accession JQ686693)
Red = BER1 (NCBI accession JQ686694)
turquoise background = differences from Refseq; numbering as Refseq

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15017  ATAATTTAATTTTATAATAAAATTTTTATCATATTATAATAATATAAAAATTTTATAAATTTATTTTTTAAATTTTACAAAATTTTTAAAATTTTTATTT
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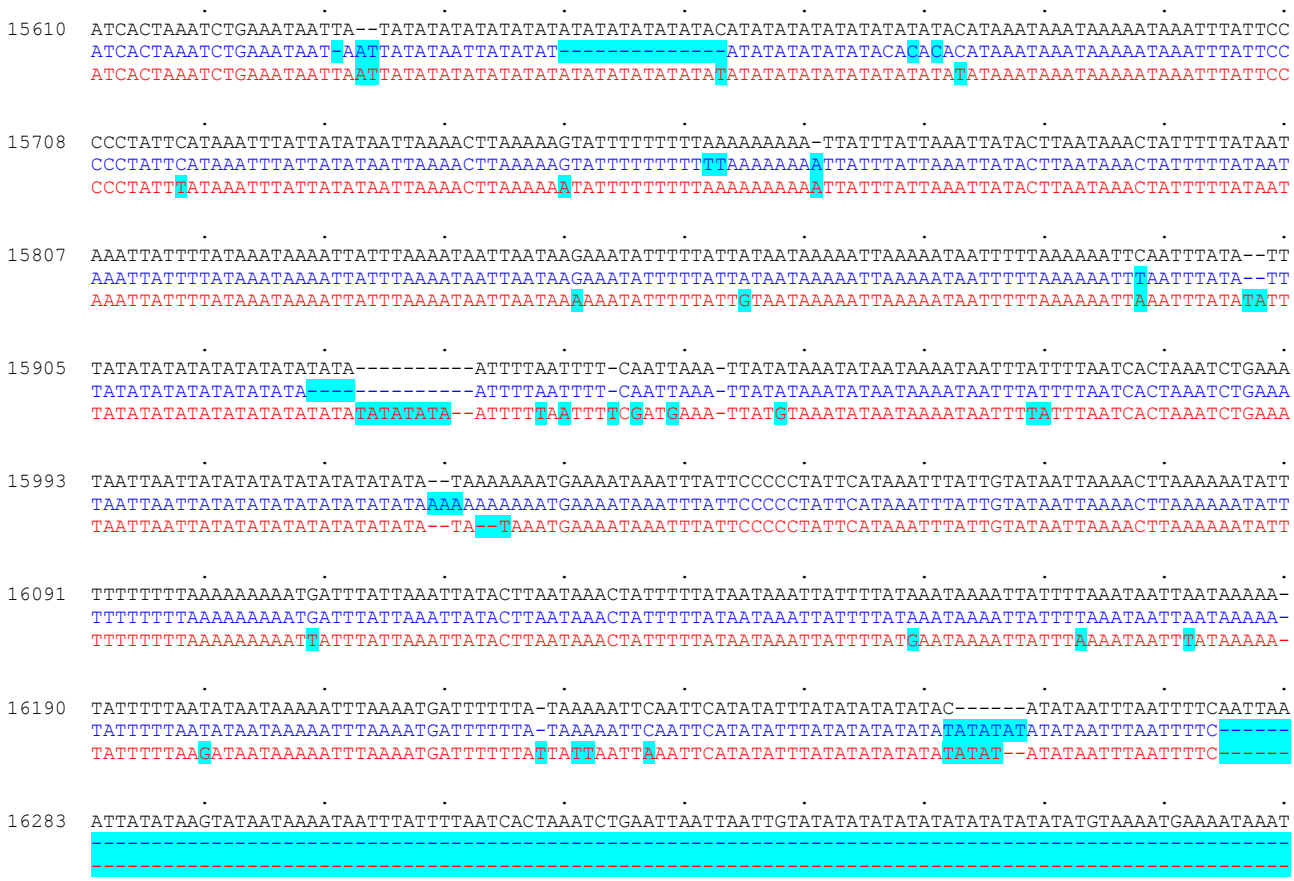

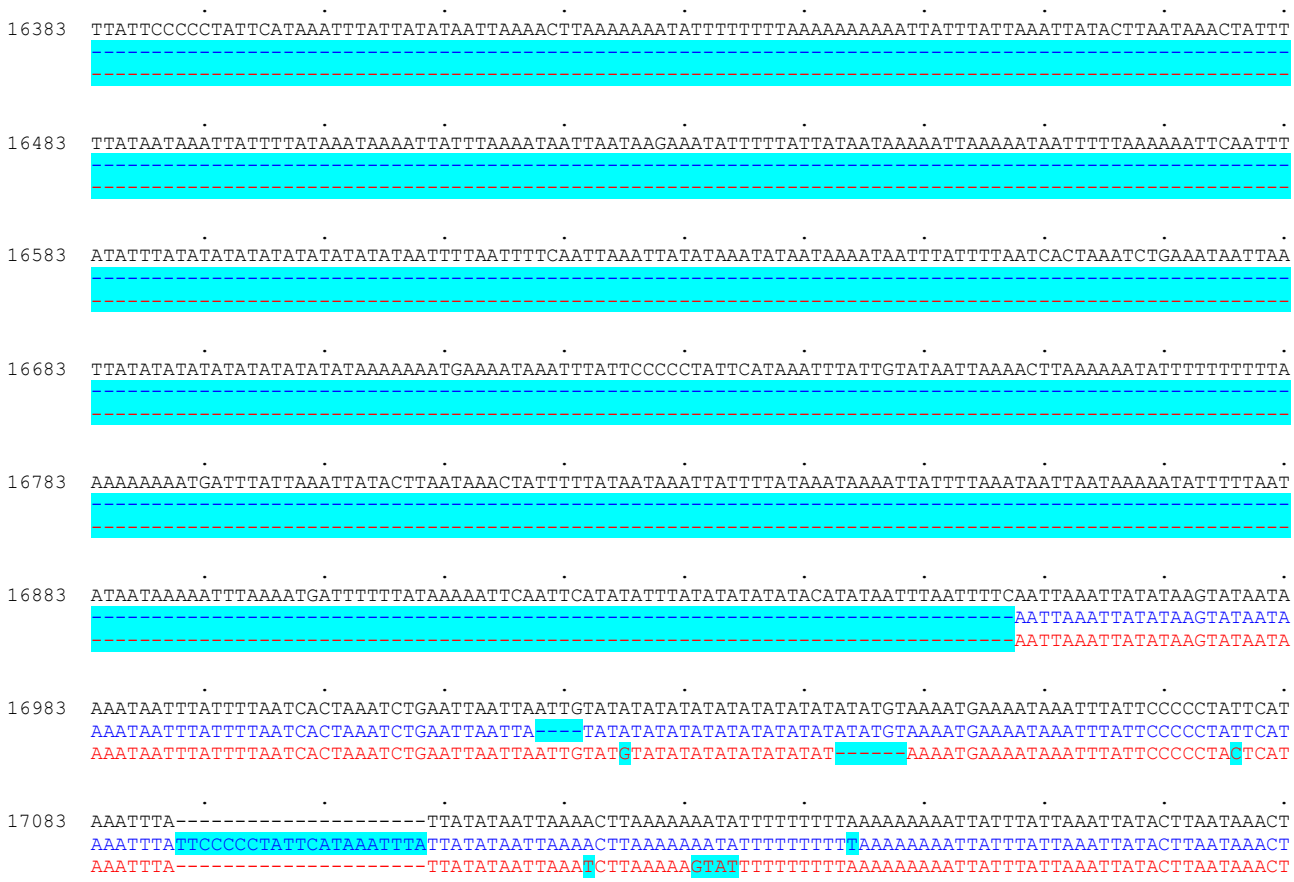
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AATTAAATTATATAAGTATAATA
16983 AAATAATTTATTTTAATCACTAAATCTGAATTAATTAATTGTATATATATATATATATATATATATGTAAAATGAAAATAAATTTATTCCCCCTATTCAT
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17083 AAATTTA----------TTATATAATTAAAACTTAAAAAAATATTTTTTTTTAAAAAAAAATTATTTATTAAATTATACTTAATAAACT
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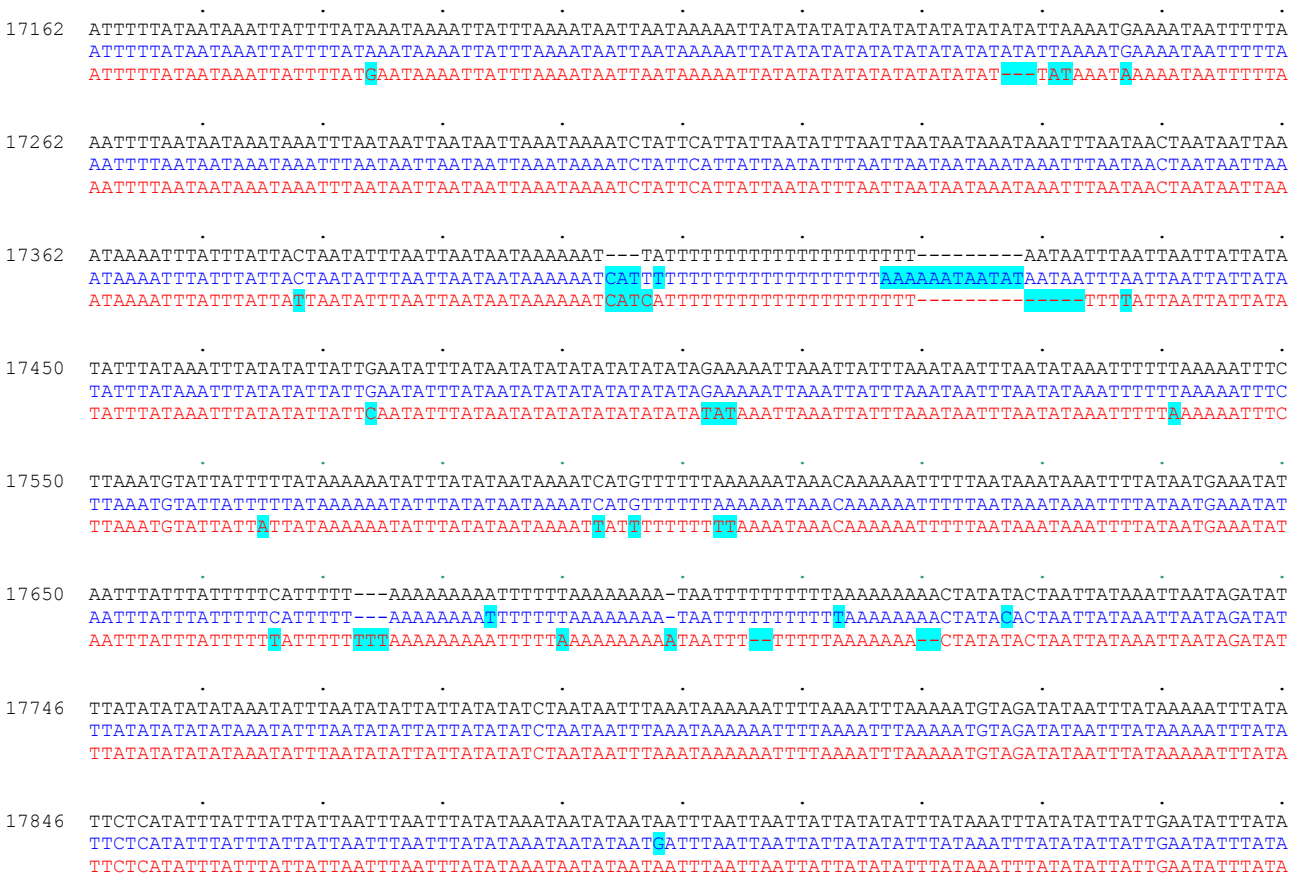

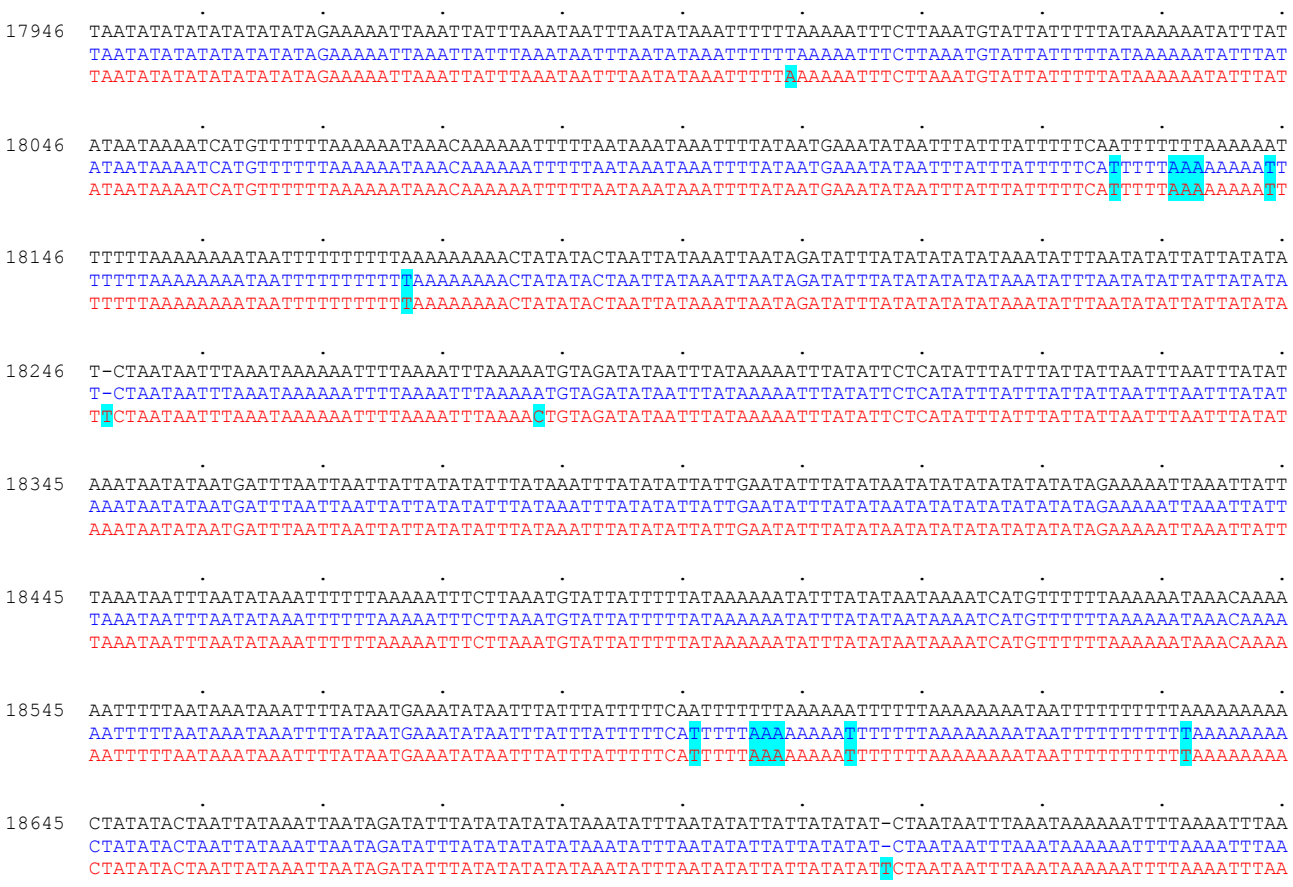
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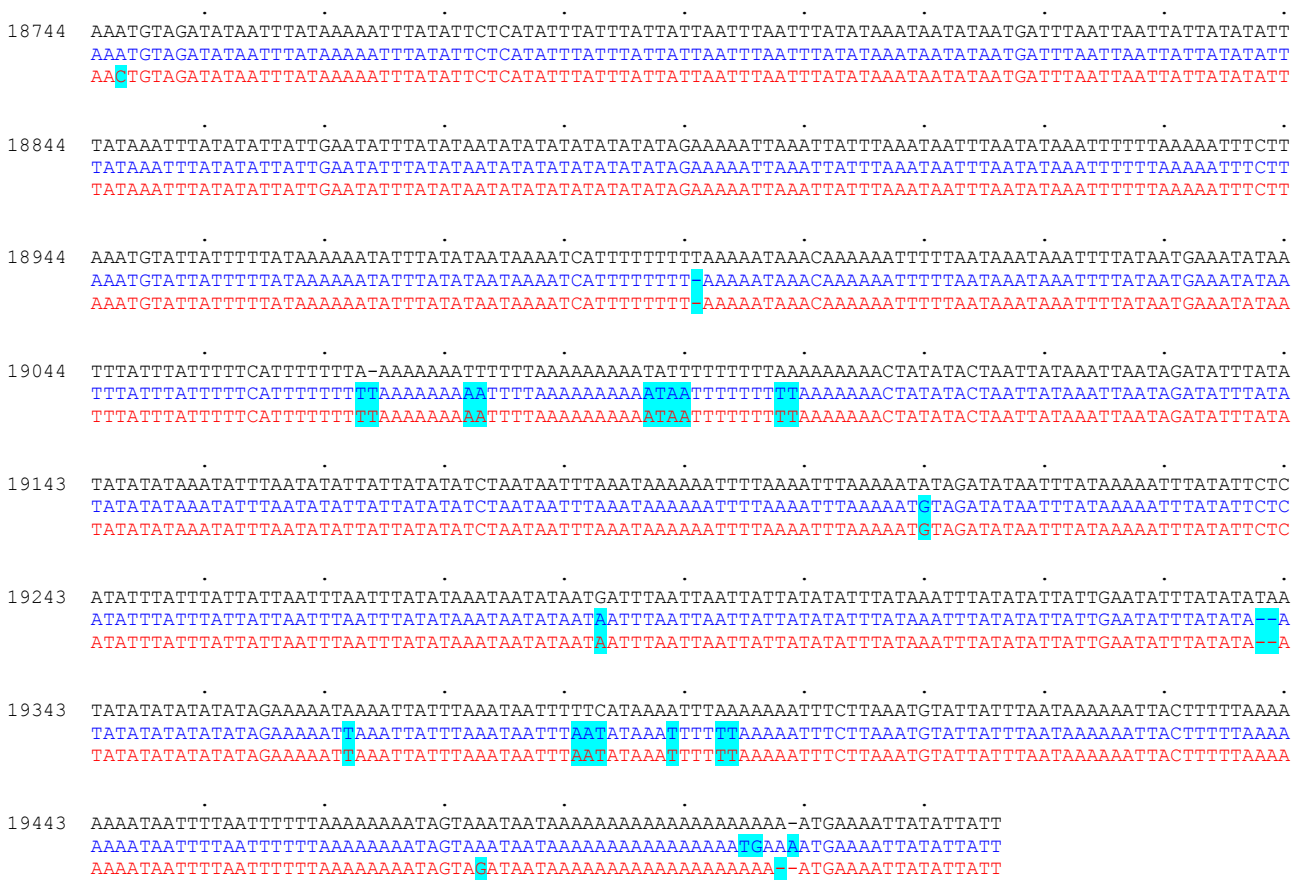
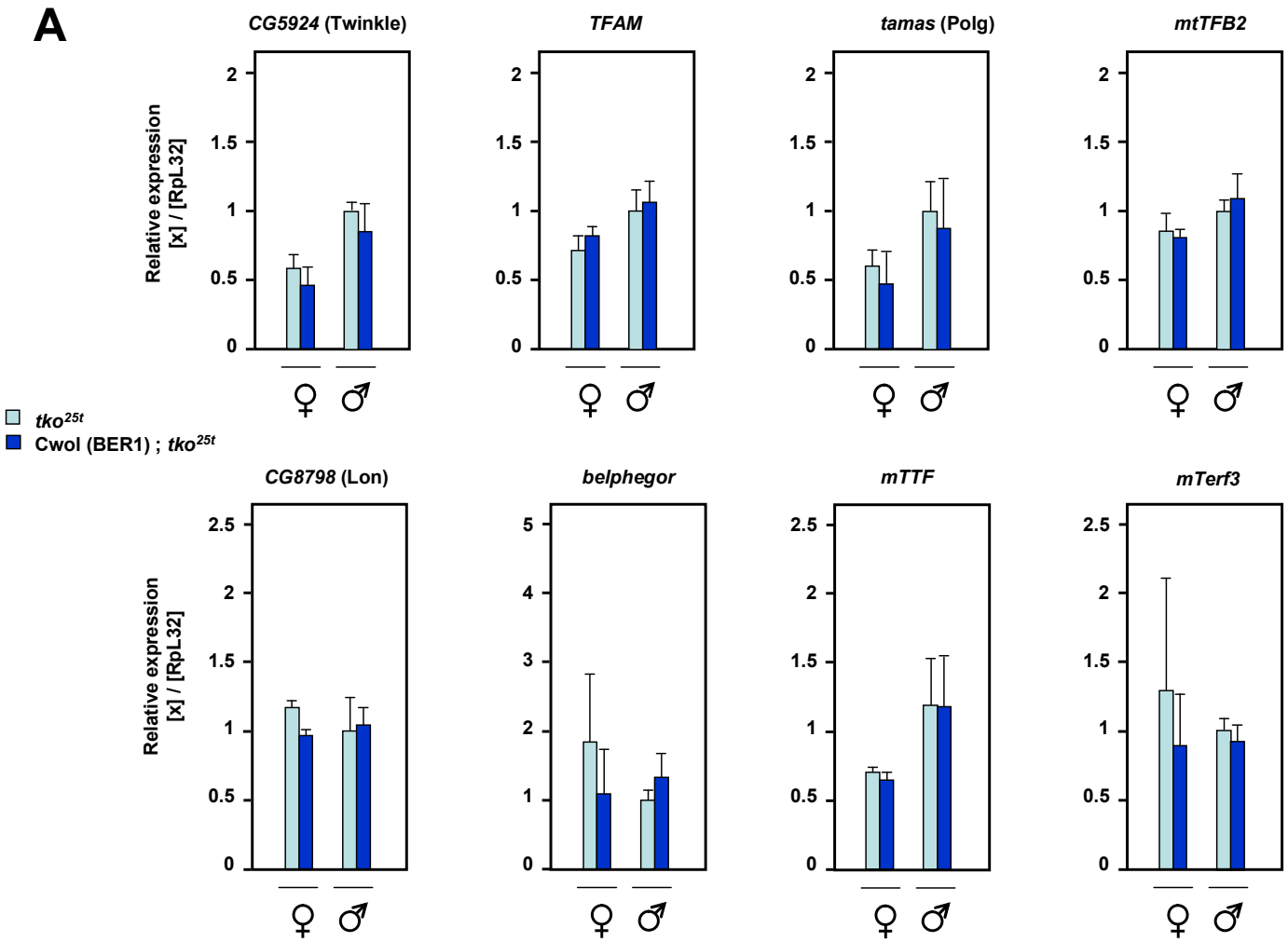


Figure S4 NCR structure and phenotypes conferred by mtDNAs of different *D. melanogaster* strains. (A) Phenotypic characterization of *tko*^{25t} flies in the Canton S and Oregon R mtDNA backgrounds, as indicated showing both to be fully of the non-suppressor type. a, b and c denote significantly different data classes (Newman-Keuls test, $p < 0.05$, based on ANOVA, $p < 0.001$, each sex considered separately). (B) Summary map of the NCR of different strains, based on crude length determination of the NCR-containing long PCR product (DsmtD4s/ DsmtD4as) by agarose gel electrophoresis (C), restriction mapping (D,E) and DNA sequencing (F), and showing the restrictions sites used in mapping. All strains studied, apart from Canton S, were found to have the 'short' NCR morph (Panel C). Partial restriction digestion (D, E) and complete sequencing (F-H) revealed that this morph contains only three copies of repeat I, rather than the 5 seen in the 'long' morph of the reference sequence (LEWIS *et al*. 1994). (D), (E) Neutral PAGE of the NCR-containing PCR product of strains BER1 and Oregon R, labeled as described in Supplementary Materials and Methods to create sub-fragments labeled at the right (tRNA-ile) or left (SSU rRNA) ends as indicated, then digested with the restriction enzymes shown (4 u, except where indicated), alongside labeled size markers. The separate sub-panels of (D) and (E) each represent sets of non-adjacent lanes from a single gel. (F, G) Sequencing strategy for the NCR, showing restriction sites in repeat array I used for cloning, and repeated priming sites for PCR in repeat array II. Primers are denoted as follows: 1 - Dm15578F, 2 - Dm17295R, 3 - Dm17556F, 4 - Dm18026, 5 - Dm17717F, 6 - Dm17833R (H) Aligned sequences of the NCR of suppressor strain BER1 and the original *tko*^{25t} non-suppressor strain, numbered according to the reference sequence (Refseq) and color-coded as indicated. Gaps indicated by dashes.


A
CG5924 (Twinkle)
TFAM
tamas (Polg)
mtTFB2
CG8798 (Lon)
belphegor
mTTF
mTerf3
Relative expression
[x] / [RpL32]
tko25t
Cwol (BER1) ; tko25t
♀
♂

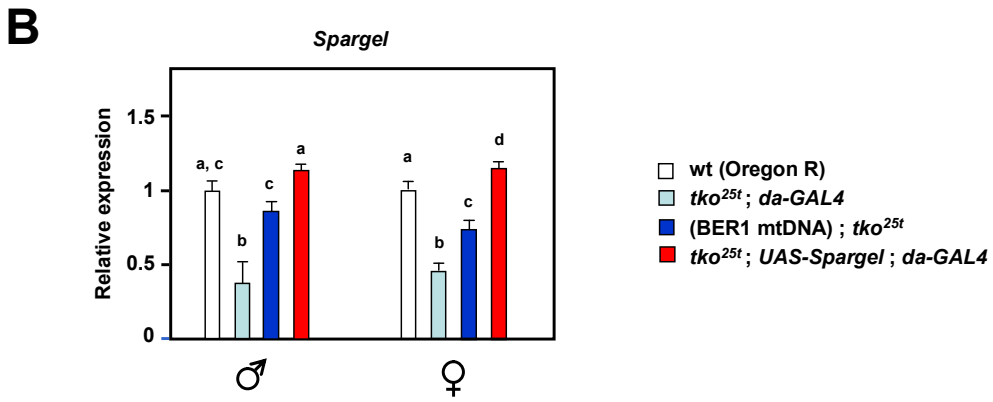


Figure S5 Expression levels of genes with known or proposed roles in mtDNA metabolism in *tko*^{25t} flies, in different mtDNA backgrounds. Q-RT-PCR of the indicated mRNAs, normalized to that of *RpL32* and then to the values for *tko*^{25t} females in the original (Oregon R-related) strain background, in flies of the strains and sex indicated. (A) Despite varying expression levels in males versus females, there were no significant differences between *tko*^{25t} flies of a given sex in the two mtDNA backgrounds tested (*t* test, $p > 0.05$ in all cases). (B) Use of the *UAS-Spargel* transgene, in combination with the ubiquitous *da-GAL4* driver, over-rode the downregulation of *Spargel* in *tko*^{25t} flies. a, b, c, d denote significantly different data classes (Newman-Keuls test, $p < 0.05$, based on ANOVA, $p < 0.01$)

Table S1 Sequence polymorphism in mtDNA coding region of suppressor and non-suppressor strains compared with Refseq

Nucleotide change	Gene	Coding change	BER1	QI2	CO3	Reids-1	*tko*^{25t} (original)	Oregon R-C	Canton S	Oregon R
671C>T	ND2	silent	X						X	
710G>A	ND2	silent	X						X	
735C>T	ND2	silent	X						X	
791A>G	ND2	silent	X						X	
838TA>AT	ND2	L>Y	X	X	X	X	X	X	X	X
1068A>C	ND2	I>L	X						X	
1154T>C	ND2	silent							X	
1478G>A	COI	R>Q					X			
1512T>C	COI	silent							X	
1674G>A	COI	silent	X						X	
1779A>T	COI	silent	X						X	
1836A>G	COI	silent	X						X	
1861T>C	COI	silent	X						X	
1917A>G	COI	silent	X							
1929G>A	COI	silent	X						X	
2071C>T	COI	silent	X							
2136G>A	COI	silent		X	X					
2160T>C	COI	silent	X						X	
2187C>T	COI	silent	X						X	
2661C>T	COI	silent	X							
2863C>T	COI	silent	X	X	X	X	X	X	X	X
2928A>T	COI	Q>H				X				
2964G>A	COI	silent	X						X	
3517T>C	COII	silent	X						X	
3583T>C	COII	silent	X							
3685G>A	COII	silent	X						X	
4088C>T	A6	silent					X	X		X
4096T>C	A6	L>S	X	X	X	X	X	X	X	X
4247A>T	A6	silent	X						X	
4592T>A	A6	N>K					X	X		X
4599T>C	A6	S>P	X						X	
4616A>T	A6	silent							X	
4620A>G	A6	M>V	X						X	
4694G>A	A6	silent				X				
4762C>T	COIII	silent	X						X	
4898G>A	COIII	V>M				X				

5212A>G	COIII	silent	X						X	
5347C>T	COIII	silent	X							
5396T>C	COIII	silent		X	X	X	X	X	X	X
5419C>T	COIII	silent		X	X	X	X	X		X
5524A>G	COIII	silent		X	X	X				
5644C>T	ND3	silent		X	X	X	X	X		X
5964delAT	intergenic			X	X					
5966delAT	intergenic			X	X	X	X	X	X	X
5966ATT>TTATA	intergenic		X							
6050insTTAAT	intergenic								X	
6301A>C	tRNA-glu	TΨCloop							X	
6305C>T	tRNA-glu	TΨCloop	X	X	X	X	X	X	X	X
6620A>T	ND5	I>M		X	X	X	X	X		X
6982A>G	ND5	silent		X	X	X	X	X	X	X
7130delCAATTC	ND5	delIG	X	X	X	X	X	X		X
7180G>A	ND5	silent	X						X	
7870G>A	ND5	silent	X							
7888G>A	ND5	silent	X						X	
7906T>C	ND5	silent					X	X		X
8153T>A	tRNA-his	DHU loop	X							
8875G>A	ND4	silent							X	
8944AC>GA	ND4	Y>H	X	X	X	X	X	X	X	X
8981C>T	ND4	silent	X							
9005A>G	ND4	silent	X						X	
9044T>A	ND4	silent	X						X	
9064C>A	ND4	V>L							X	
9187A>G	ND4	silent	X						X	
9783G>A	ND4L	silent	X				X	X		X
9886delT	tRNA-thr	TΨCloop					X	X		X
9990C>T	ND6	silent	X						X	
10225C>T	ND6	silent	X							
10289C>A	ND6	S>Y				X				
10389C>T	ND6	silent	X						X	
10905A>G	Cyt b	Silent	X							
10925T>G	Cyt b	V>G	X	X	X	X	X	X	X	X
10935T>A	Cyt b	silent	X	X	X	X	X	X	X	X
10952AC>CA	Cyt b	Y>S	X	X	X	X	X	X	X	X
11265C>T	Cyt b	silent	X		X				X	
11279C>A	Cyt b	T>N	X	X	X	X	X	X	X	X
11560T>C	Cyt b	silent	X						X	
11893T>A	ND1	I>F	X	X	X	X	X	X	X	X

11916GG>CC	ND1	P>G	X	X	X	X	X	X		
11987C>A	ND1	L>F	X	X	X	X	X	X	X	X
12060T>G	ND1	N>T	X	X	X	X	X	X	X	X
12062C>A	ND1	silent					X	X		X
12091C>T	ND1	V>M	X						X	
12131T>C	ND1	silent		X	X				X	
12316A>G	ND1	silent		X	X					
12344A>C	ND1	silent							X	
12380C>T	ND1	silent							X	
12626T>C	ND1	silent	X	X	X	X	X	X	X	X
12804T>C	LSU rRNA			X	X	X	X	X		X
13065delA	LSU rRNA			X	X	X	X	X		X
13289T>A	LSU rRNA		X	X	X	X	X	X	X	X
13295delA	LSU rRNA		X	X	X	X	X	X	X	X
13561C>T	LSU rRNA								X	
13587G>A	LSU rRNA							X		X
14698T>C	SSU rRNA			X	X					

Notes:

All numbering based on Refseq (NCBI accession NC_001709). Oregon R sequence from NCBI accession AF200828

New database submissions: *tko*^{25t} original strain, full genome sequence, NCBI accession JQ686693 ; BER1 full genome sequence, NCBI accession JQ686694; Oregon R-C full genome sequence, NCBI accession JQ686698; CO3 coding region sequence, NCBI accession JQ686695;
QI2 coding region sequence, NCBI accession JQ686696; Reids-1 coding region sequence, NCBI accession JQ686697; Canton S coding region sequence, NCBI accession JQ686699

Yellow background denotes strain-specific polymorphisms

Blue background denotes polymorphisms over-represented in suppressor cytoplasms

Grey background denotes polymorphisms over-represented in nonsuppressor cytoplasms

Significant heteroplasmy is excluded, based on the following: our sequencing strategy involved pooling of multiple PCR products, followed by sequencing on both strands using an overlapping primer set. All novel polymorphisms were verified by additional PCR and sequencing. In no case did we find any evidence for heteroplasmy, within the limits of detection by Sanger sequencing (10-15%). Disease-associated heteroplasmy in humans is easily detected by such sequencing, with relative levels of pathological mutant mtDNA in the 50-90% range. Thus we can categorically rule out any significant contribution of heteroplasmy to the *tko*^{25t} phenotype, in any of the strains tested. Moreover, *tko*^{25t} breeds true phenotypically of over many generations in either the suppressor or non-suppressor background, showing standard X-linked recessive inheritance, which would not be expected if the phenotype were subject to influences of heteroplasmy. Conversely, the suppressor itself shows standard maternal inheritance, which would also not be expected if heteroplasmy were a significant factor in suppression.

Table S2 Primers used for PCR and sequencing

Target gene	Primer name	Sequence (5´ to 3´)	Purpose
CG5924 (Twinkle)	CG5924 left	GCATCGTAGTGCAACCAAAA	Q-RT-PCR
	CG5924 right	CCAAAGCGGTTCTAGTCAGC	
mtTFB2	mtTFB2 left	CAGGATCTACCCGCTCTCTG	Q-RT-PCR
	mtTFB2 right	AGATGGGTGTTACGGACTCG	
tamas (Polg)	tamas left	AATCTCTTCCAGGCGATTGA	Q-RT-PCR
	tamas right	CAAAGGGCAAGCGAGTGTA	
Tfam	Tfam left	GGCTCAGGTGGATCGATAAG	Q-RT-PCR
	Tfam right	GAGTGGCACCAAAAGACCAC	
mTTF	mTTF left	AGTTCAGAGCACCCACCAGT	Q-RT-PCR
	mTTF right	ACTGCAGCTAGAGGGCGTTA	
mTerf3	mTerf3 left	CGTTCCCGCAGTCTAAATTC	Q-RT-PCR
	mTerf3 right	CGTTCCCGCAGTCTAAATTC	
CG8798 (Lon)	CG8798 left	GTTTCAGTGGCCTTCTCCAG	Q-RT-PCR
	CG8798 right	AAAGTACCGCGAAAAGCTGA	
belphegor	Belpgegor-F	GCCTCTTGCGCTTGTACT	Q-RT-PCR
	Belpgegor-R	TTCGAACACGTCTTTCCG	
Ets97D	Delg-F	TGATGGATTCATGGATGACG	Q-RT-PCR
	Delg-R	AGAATCATGTCGGCCAATTC	
Spargel	Sparg-F	CCTCGACTACATTCGGTGCT	Q-RT-PCR
	Sparg-R	AGACGTGCCTTCTGTCGTTC	

LSU rRNA	16S-L	TGGCCGCAGTATTTTGACTG	Q-RT-PCR
	16S-R	TCGTCCAACCATTCATTCCA	
SSU rRNA	12S-L	AAAAATTTGGCGGTATTTTAGTCT	Q-RT-PCR
	12S-R	AAGGTCCATCGTGGATTATCG	
RpL32	Rp49-f	AGCATACAGGCCCAAGATCGTGAA	Q-RT-PCR
	Rp49-r	CACGTTGTGCACCAGGAACTTCTT	
mtDNA	DsmtD1s	GTTTTCTGCATTCATTGACTGATTTATA	PCR of coding region (CR) fragment 1
	DsmtD1as	TTTGACATTGAAGATGTTATGGAGATTA	
	DsmtD2s	GAGAAGGAACATACCAAGGATTACATAC	PCR of coding region (CR) fragment 2
	DsmtD2as	GAGTTAAAGTGGCATTATCAACAGCAAA	
	DsmtD3s	TCCGATTAGAAACAAAACAAAATAGCCC	PCR of coding region (CR) fragment 3
	DsmtD3as	AAAGTATTGACTAAATTGGTGCCAGCAG	
	DsmtD4s	ATCTTACCTTAATAATAAGAGCGACGGG	PCR of NCR containing fragment
	DsmtD4as	TTAGGAAATC AAAAATGGAA AGGAGCGG	
	Dm189F	AGCTACTGGGTTCATACCCC	Sequencing of CR fragment 1
	Dm710F	GGTTATTATTGGAGCTATTGGAGG	Sequencing of CR fragment 1
	Dm994R	GGAGGTAATCCTCCTAATGATAA	Sequencing of CR fragment 1
	Dm1274F	GTTAATAAAACTAATAACCTTCAAAGC	Sequencing of CR fragment 1
	Dm1480R	GTCGCGATTATTGATTAAGTG	Sequencing of CR fragment 1
	Dm1777R	GTCAAAATCTTATATTATTTATTCGTG	Sequencing of CR fragment 1
	Dm1825F	AATGGAGCTGGAACAGGATG	Sequencing of CR fragment 1
	Dm2079R	TCCTGCTAGTACTGGAAGTG	Sequencing of CR fragment 1
	Dm2371F	CGAGCTTATTTTACCTCAGC	Sequencing of CR fragment 1
	Dm2659R	GGTATCAGTGAATAAAACCTGC	Sequencing of CR fragment 1
	Dm2896F	GTATCACAACGACAAGTAATTTACC	Sequencing of CR fragment 1
	Dm3125R	GAGAAGCTCTATCTTGTAAACC	Sequencing of CR fragment 1

Dm3277F	AACTATTTTACCAGCAATTATTTTACT	Sequencing of CR fragment 1
Dm3524R	AGTTTATAGGTAAAACTACTCGG	Sequencing of CR fragment 1
Dm3778F	CTGAAAGCAAGTACTGGTCTC	Sequencing of CR fragment 1
Dm3780R	CAGTCATCTAATGAAGAGTTATTTCTA	Sequencing of CR fragment 1
Dm4100R	AGCTAAGGGGTCGAATACAG	Sequencing of CR fragment 1
Dm4244F	AGGACCATCAGGTCATAATGG	Sequencing of CR fragment 1
Dm4467R	CGGGTGTTCCTTGAGGAAC	Sequencing of CR fragment 1
Dm4743F	CACACTCAAATCACCCTTTCC	Sequencing of CR fragment 1
Dm5064R	GCGGGTGATAAACTTCTGTG	Sequencing of CR fragment 1
Dm5289F	CTCCATTTACTATTGCAGACTC	Sequencing of CR fragment 1
Dm5521R	TCCTCCTCATCAGTAAATTGTG	Sequencing of CR fragment 1
Dm5740F	CCAAAATCTTCATCTCGATTACC	Sequencing of CR fragment 1
Dm6074R	CAATCAATCGCTTCATATTCAG	Sequencing of CR fragment 1
Dm5314F	ACTGTAACTTGAGCCCACCA	Sequencing of CR fragment 2
Dm6005F	TTGATTGCAATTAGTTTCGACCT	Sequencing of CR fragment 2
Dm6195R	CATTAACAGTGATACGCCTC	Sequencing of CR fragment 2
Dm6801F	AAATCAATCAATTTAATATTCTACCTC	Sequencing of CR fragment 2
Dm6928R	CGGTGATTTAAATTGCGGTAG	Sequencing of CR fragment 2
Dm7191F	GCCCCAGCACATATAAACAA	Sequencing of CR fragment 2
Dm7378R	ATTAACAATATTTATAGCTGGATTAGG	Sequencing of CR fragment 2
Dm7771F	AAACAAGTCCTAAACCATCTCACC	Sequencing of CR fragment 2
Dm8181R	AATTTGTGGTGTTAGTGATATGAAAA	Sequencing of CR fragment 2
Dm8740F	TGAGCAACAGATGAATAAGCAA	Sequencing of CR fragment 2
Dm8762R	TTGCTTATTCATCTGTTGCTCA	Sequencing of CR fragment 2
Dm9363F	AATCCATAAGATAATATATCACAACCT	Sequencing of CR fragment 2
Dm9623R	ATGTGAAGGGGCCTTAGGTT	Sequencing of CR fragment 2

	Dm9888R	ATAATCTTATTTTTGATTTACAAGACC	Sequencing of CR fragment 2
	Dm10196R	TCATTAGAGGCTAAAGATGTTAC	Sequencing of CR fragment 2
	Dm10525R	TGGGAATTTCGTAAAGGTTTATTC	Sequencing of CR fragment 2
	Dm9858F	CATTGGTCTTGTAAATCAAAAATAAG	Sequencing of CR fragment 3
	Dm10196R	TCATTAGAGGCTAAAGATGTTAC	Sequencing of CR fragment 3
	Dm10465F	TTTAAAGGACCTATTCGAATAATATC	Sequencing of CR fragment 3
	Dm10725R	ATAATTAACGTCTCGACAAATATG	Sequencing of CR fragment 3
	Dm10950F	ATACGCTATCCCTTACTTAGG	Sequencing of CR fragment 3
	Dm11258R	GGGTCTCCCAATAAATTTGGTC	Sequencing of CR fragment 3
	Dm11425F	TTAAGAAAATTCCGAGGGATTC	Sequencing of CR fragment 3
	Dm11845R	GGAACTTTACCTCGATTTCG	Sequencing of CR fragment 3
	Dm12075F	GCTAATGAAATAGATACTCAAACTAAA	Sequencing of CR fragment 3
	Dm12244R	GCTGTGGCTCAGACTATTTC	Sequencing of CR fragment 3
	Dm12492F	GCATCACAAAAAGGTTGAGG	Sequencing of CR fragment 3
	Dm12584R	TTTATTAGAACGAAAAGTTTTAGGATA	Sequencing of CR fragment 3
	Dm12734R	AACTATTTTGGCAGATTAGTGC	Sequencing of CR fragment 3
	Dm12976F	CGCTGTTATCCCTAAAGTAAC	Sequencing of CR fragment 3
	Dm13172R	AGACGAGAAGACCCTATAAATC	Sequencing of CR fragment 3
	Dm13390F	GGCGAATATTATTTTTGCCG	Sequencing of CR fragment 3
	Dm13661R	ATAATTTTAATGTTTTATGGGATAAGC	Sequencing of CR fragment 3
	Dm13852F	TATTTAATAAACACTGATACACAAGGT	Sequencing of CR fragment 3
	Dm14152R	CTGGAAAGTGTATCTAGAATGAC	Sequencing of CR fragment 3
	Dm14332F	AATATAAGCTACACCTTGATCTG	Sequencing of CR fragment 3
	Dm14366R	AAAAATTTATATCAGATCAAGGTGTAG	Sequencing of CR fragment 3
	Dm14502R	CGGTATTTTAGTCTATCTAGAGG	Sequencing of CR fragment 3
	Dm14428F	TGATTACAAATTTAAGTAAGGTCCATCG	PCR and sequencing of ‘left’ end of

			NCR and of adjacent CR segment
	Dm14570F	AGGGTATCTAATCCTAGTTT	Sequencing of 'left' end of NCR and of adjacent CR segment
	Dm14721F	AATGGTATAACCGCGACTGC	Sequencing of 'left' end of NCR and of adjacent CR segment; PCR of NCR repeat array I
	Dm14787R	CCAAATTGGTGCCAGCAGTCGCGG	Sequencing of 'left' end of NCR and of adjacent CR segment
	Dm15285F	AAAAAATTATAGATTAATTTCTTTTAAATGAC	Sequencing of 'left' end of NCR and start of repeat array I
	Dm15578F	CGAATAATAAATATAATAAATAATTTATTTTAATCACTAAATCTG	PCR of NCR repeat arrays I and II and region between them
	Dm17295R	GAATAGATTTTATTTAAT	PCR of NCR repeat array I; sequencing of region between repeat arrays I and II
	Dm17556F	GTATTATTTTTATAAAAAATATTTATATAATAAAATCATG	PCR and sequencing of NCR repeat array II

	Dm17556R	CATGATTTTATTATATAAATATTTTTTATAAAAATAATAC	PCR of NCR repeat array I; PCR and sequencing of region between repeat arrays I and II
	Dm17717F	ACTATATACTAATTATAAATTAATAG	PCR and sequencing of NCR repeat array II
	Dm17833R	GAGAATATAAATTTTTATAAATTATATC	PCR and sequencing of NCR repeat array II and region between repeat arrays I and II
	Dm18026R	ATAATACATTTAAGAAATTTTTAAAAAATTTATATT	PCR and sequencing of NCR repeat array II
	Dm18933F	AAAATTTCTTAAATGTATTATTTAATAAAAAATTACTTTTTAA	PCR of repeat array II, 'right' end of NCR and adjacent CR seqment; sequencing of NCR repeat array II
	Dm31R	CATGATTTACCCTATC	Sequencing of NCR repeat array II and 'right' end of NCR

	Dm225R	TATAACCTTTATAAATGGGGTATGAACCCAGTAG	PCR of whole NCR and of repeat array II Sequencing of NCR repeat array II, 'right' end of NCR and adjacent CR segment
Wolbachia 16S rRNA	w-16SF	TTGTAGCCTGCTATGGTATAACT	PCR of *Wolbachia* genomic DNA for detection assay
	w-16sR	GAATAGGTATGATTTTCATGT	
Universal 16S rRNA (bacterial)	Eub-16SF	GCTTAACACATGCAAG	PCR of bacterial genomic DNA for detection assay
	Eub-16SR	CCATTGTAGCACGTGT	
LSU rRNA (mt)	mt 16S-F	TTCGTCCAACCATTCATTCC	Q-PCR for copy number assay
	mt 16S -R	TTTGTCTAACCTGCCCACTGA	
18S rRNA (nuclear)	18S-F	TTGCGAAACAACCGTAACAC	Q-PCR for copy number assay
	18S-R	GGTAAACCGCTGAACCACTT	

All sequences are shown 5′ to 3′