A Cytoplasmic Suppressor of a Nuclear Mutation Affecting Mitochondrial Functions in *Drosophila*

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ABSTRACT Phenotypes relevant to oxidative phosphorylation (OXPHOS) in eukaryotes are jointly determined by nuclear and mitochondrial DNA (mtDNA). Thus, in humans, the variable clinical presentations of mitochondrial disease patients bearing the same primary mutation, whether in nuclear or mitochondrial DNA, have been attributed to putative genetic determinants carried in the "other" genome, though their identity and the molecular mechanism(s) by which they might act remain elusive. Here we demonstrate cytoplasmic suppression of the mitochondrial disease-like phenotype of the *Drosophila melanogaster* nuclear mutant *tko*^{25t}, which includes developmental delay, seizure sensitivity, and defective male courtship. The *tko*^{25t} strain carries a mutation in a mitoribosomal protein gene, causing OXPHOS deficiency due to defective intramitochondrial protein synthesis. Phenotypic suppression was associated with increased mtDNA copy number and increased mitochondrial biogenesis, as measured by the expression levels of *porin* voltage dependent anion channel and *Spargel* (PGC1α). Ubiquitous overexpression of *Spargel* in *tko*^{25t} flies phenocopied the suppressor, identifying it as a key mechanistic target thereof. Suppressor-strain mtDNAs differed from related nonsuppressor strain mtDNAs by several coding-region polymorphisms and by length and sequence variation in the noncoding region (NCR), in which the origin of mtDNA replication is located. Cytoplasm from four of five originally *Wolbachia*-infected strains showed the same suppressor effect, whereas that from neither of two uninfected strains did so, suggesting that the stress of chronic *Wolbachia* infection may provide evolutionary selection for improved mitochondrial fitness under metabolic stress. Our findings provide a paradigm for understanding the role of mtDNA genotype in human disease.

THE oxidative phosphorylation (OXPHOS) system depends upon the cooperative expression of genes in nuclear and mitochondrial DNA (mtDNA). In most metazoans, including humans and *Drosophila*, the mitochondrial genome encodes 13 essential polypeptides of the OXPHOS complexes of the inner mitochondrial membrane, plus the two rRNAs and 22 tRNAs required for their synthesis inside mitochondria. The remaining polypeptides of the OXPHOS complexes, as well as all of the proteins required for mtDNA maintenance and expression, are nuclear coded.

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Mitochondrial disease in humans takes many forms ranging from rare neurological syndromes to cases of common multifactorial disorders (Greaves *et al.* 2012; Ylikallio and Suomalainen 2012). Pathological OXPHOS mutations can be in nuclear or mitochondrial genes, and these have also been hypothesized to interact to modify disease phenotype (Brown *et al.* 1997, 2002; Hudson *et al.* 2007). Variants of mtDNA have also been postulated to be under evolutionary selection, in a complex interaction with the nuclear genome and environmental factors (Willett and Burton 2004; Wallace 2005; Meiklejohn *et al.* 2007). Although direct evidence for a modifier effect of mtDNA in disease is lacking, naturally occurring variants have been experimentally shown to have effects on OXPHOS biochemistry (Katewa and Ballard 2007), reactive oxygen species (ROS) production (Moreno-Loshuertos *et al.* 2006), and other aspects of cellular (Kazuno *et al.* 2006) and organismal physiology (Ballard *et al.* 2007a; Pravenec *et al.* 2007).

Drosophila provides an ideal model system in which the effects of mtDNA variation on OXPHOS disease can be directly tested. The *tko*^{25t} strain, bearing a point mutation in the gene encoding mitoribosomal protein S12 (Toivonen *et al.* 2001), shows an organismal phenotype of developmental delay, bang sensitivity, impaired sound response, and defective male courtship, associated with severe deficiency of the four OXPHOS complexes (I, III, IV, and V) containing mitochondrial translation products (Shah *et al.* 1997). The primary effect of the mutation appears to be on the stability or assembly of the mitoribosomal small subunit, based on measurements of rRNA levels (Toivonen *et al.* 2001, 2003). We previously determined that the mitochondrial disease-like phenotype of *tko*^{25t} is partially suppressible by duplication of the *tko* locus (Kemppainen *et al.* 2009), although mtDNA effects were not analyzed in earlier studies.

In *Drosophila*, the frequent presence of the endosymbiont *Wolbachia*, one of the closest extant relatives of the mitochondrial ancestor, provides an additional dimension to nuclear–cytoplasmic genetic interactions, especially since its presence is correlated with mtDNA copy-number variation (Ballard and Melvin 2007). *Wolbachia* has elsewhere been reported to influence susceptibility to viral pathogens Hedges *et al.* 2008; Teixeira *et al.* 2008), manipulate lifespan (Fry and Rand 2002; Reynolds *et al.* 2003; Toivonen *et al.* 2007), and increase insulin signaling (Ikeya *et al.* 2009). We therefore set out to determine whether *Wolbachia*-containing cytoplasm could modify the mitochondrial phenotype of *tko*^{25t} and whether any such effect depended on the actual presence of *Wolbachia*, on coinherited mtDNA variants, or the combination.

Materials and Methods

Drosophila stocks and maintenance

tko^{25t} flies in the original strain background were maintained at room temperature as balanced stock (FM7 balancer), as previously shown (Toivonen *et al.* 2001). Introgression into other cytoplasmic backgrounds was performed essentially as shown in Supporting Information, Figure S1B, with generally five or more generations of backcrossing before balanced stocks were reconstituted, which were then used to generate *tko*^{25t} homozygotes and hemizygotes for individual experiments. *sesB*^{1} flies, also in the original Oregon R background (Homyk and Sheppard 1977; Zhang *et al.* 1999), obtained from M. Ashburner, University of Cambridge, Cambridge, UK, were balanced, maintained, and manipulated similarly. The *UAS-Spargel* (UAS-*Srl*) line, kindly supplied by C. Frei, ETH, Zurich, contains an insertion on chromosome 2 of the coding region of the *Spargel* gene under upstream activation sequence (UAS) promoter control (Tiefenböck *et al.* 2010), balanced with CyO. Wild-type strains BER1, KSA2, Oregon R-C, Reids-1, CO3, QI2, BS1, and M2 were obtained from the Bloomington Stock Center. Standard lab strains Canton-S and Oregon R, and the da-GAL4 driver strain (Wodarz *et al.* 1995) have been maintained in our own laboratories for >10 yr. Prior to testing for *Wolbachia* infection, or when flies were found to be infected, flies were maintained in quarantine from all other stocks. Infected flies were cured of *Wolbachia* infection by growth for two generations at room temperature on medium containing 30 μg/ml tetracycline (Dobson *et al.* 2002) and then retested (Figure S1A). Flies were maintained in standard medium with supplements as previously shown (Toivonen *et al.* 2001) and reared for experiments at 25° except where stated.

Developmental time

Groups of eight virgin females and five males of a given strain or genotype were mated 5 days after eclosion for 3 days, being tipped to a fresh vial each day and then discarded. Each experiment used batches of five identical vials, and each was repeated, to generate means ± SD of the eclosion day as shown in the figures. Wild-type (Oregon R) flies were included as a control in every experiment.

Behavioral and lifespan assays

For behavioral analysis, flies were collected under CO_2 anesthesia within 2 days of eclosion and then kept for up to a further 5 days in vials with 15 flies of the same sex per vial, and transferred to fresh food vials every 2 days. Bang sensitivity and courtship were measured at room temperature, as previously shown (Toivonen *et al.* 2001). For bang sensitivity, 20–25 individual flies of each sex and genotype to be tested were used in each experiment. For courtship, at least 15 mating pairs of the genotypes to be tested were used in each experiment, which was conducted in triplicate. Courtship was scored as the proportion of females that mated within 1 hr. Lifespan was measured as previously shown (Sanz *et al.* 2010a,b), using groups of 150–300 flies of each sex, collected within 24 hr of eclosion, and kept at a density of 20 flies per vial at 25°C in a controlled 12 hr light–dark cycle. Vials were changed every 2–3 days and the number of dead flies was counted. Each independent experiment was repeated twice: data were pooled and analyzed together.

DNA extraction and quantitation

For mtDNA copy-number analysis, total DNA was prepared from batches of 10–30 adult flies, homogenized in 500 μl ice-cold homogenization medium (75 mM NaCl, 25 mM EDTA pH 8.0) in a 1.5-ml Eppendorf tube, using a motor-driven polypropylene pestle (VWR). Following addition of 70 μl 10% SDS, 200 μg proteinase K (Fermentas), and 100 μg DNase-free, proteinase-free RNase A (Fermentas) homogenates were incubated overnight at 37° with gentle shaking, followed by two extractions with phenol-chloroform-isoamyl alcohol (25:24:1) for 1 hr with gentle shaking. Extracts were centrifuged at room temperature for 15 min at 5000 g_{max}. DNA was recovered from the final aqueous phase by ethanol precipitation and resuspended in 200 μl TE buffer (pH 8.0). Relative mtDNA copy number was measured by real-time

qPCR using primers for mitochondrial large subunit (LSU) rRNA and nuclear 18S rRNA (Table S2), in a StepOnePlus instrument (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) under the manufacturer's recommended conditions, with 20 sec of enzyme activation at 95°, followed by 40 cycles of 95° for 3 sec and 60° for 30 sec. For mtDNA sequencing and noncoding A + T region (NCR) repeat analysis, mitochondria were prepared from batches of 50–200 flies, essentially as described by Miwa *et al.* (2003) (see Supporting Information for full details). The mitochondrial pellet was resuspended in 500 μl ice-cold homogenization medium and processed for DNA isolation as above.

RNA extraction and quantitation

RNA was isolated as previously shown (Fernández-Ayala *et al.* 2009; Sanz *et al.* 2010a,b) from three biological replicate samples of each sex and genotype to be tested. Expression of mitochondrial small subunit (SSU) and LSU rRNAs and of various nuclear gene transcripts was measured relative to that of the housekeeping gene *RpL32* by qRT–PCR using customized primers (see Table S2 for details), as previously shown (Fernández-Ayala *et al.* 2009; Kemppainen *et al.* 2009; Sanz *et al.* 2010a,b), together with the SYBR Green Master Mix (Applied Biosystems). Following an initial enzyme activation at 95° for 20 sec, 40 reaction cycles of denaturation for 3 sec at 95° followed by annealing and extension for 30 sec at 60° were carried out, using the StepOne real-time PCR instrument (Applied Biosystems).

Protein analysis by Western blotting

Batches of 15–20 adult (2–5 days old) flies were placed in a 1.5-ml Eppendorf tube with 100 μl PBS. An equal volume of 2× SDS–PAGE sample buffer (100 mm Tris-HCl, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mm β-mercaptoethanol, pH 6.8) was added. Samples were homogenized using a pellet pestle (VWR), heated to 100° for 5 min, centrifuged for 5 min at 12,000 g_{max}, and loaded on Criterion precast Tris-HCl 10–20% polyacrylamide gradient gel (Bio-Rad). Electrophoresis, blotting, and detection were essentially as described previously (Fernández-Ayala *et al.* 2009) (see Supporting Information for full details), except for the antibodies: primary (from Mitosciences: Porin 1:5000, ATP synthase subunit α, 1:100,000, and complex I subunit NDFUS3, 1:10,000; from Santa Cruz Biotechnology; α-actinin, 1:10,000), secondary (Vector Laboratories, peroxidase linked) antimouse IgG (H + L), or for α-actinin detection, antirabbit IgG (1:10,000). The amount of each protein was measured by densitometry with Image Lab 3.0.1 software (Bio-Rad) and normalized first to the α-actinin signal, then to the value for Oregon R wild-type flies of the given sex.

Polarography, ATP assay, and mitochondrial H_2O_2 production

Mitochondria were isolated and used to measure substrate oxidation rates at state 3 by polarography as previously described (Fernández-Ayala *et al.* 2009), using a substrate mix of 20 mM sn-glycerol 3-phosphate, 5 mM sodium pyruvate, and 5 mM proline. Mitochondrial ROS production at state 4 was assayed as previously described (Sanz *et al.* 2010a,b) by measuring Mitosox fluorescence in the absence of added ADP. ATP was assayed on batches of 15 adult females/genotype, collected at room temperature, as described previously (Fergestad *et al.* 2006), using a Harta MicrolumXS microplate luminometer with Lumiterm II acquisition software (Harta Instruments, Gaithersburg, MD) and ATP determination kit A-22066 (Invitrogen). Sample values were corrected for protein content using DC protein assays (Bio-Rad Laboratories) and ATP concentrations were normalized as a percentage of wild-type control samples.

Long PCR and mtDNA sequencing

The whole mitochondrial genome was amplified in four overlapping fragments (each ~5.5 kb in size). Three of these, representing the coding region, were sequenced using primer sets generating overlapping reads on both strands (Table S2). The NCR and its boundaries were sequenced by a strategy combining long PCR and cloning of restriction fragments (for full details see Supporting Information). Sequence analysis used the Phred/Phrap/Consed package (Gordon *et al.* 1998).

Statistical analysis

Data were analyzed by ANOVA or *t*-test, as appropriate; see figure legends for details.

Results

PCR using *Wolbachia*-specific 16S rRNA gene primers was first used to test for the presence of the endosymbiont in a set of wild-type strains collected from diverse locations and maintained at Bloomington, as well as our own stocks of tko^{25t}. While tko^{25t} was confirmed to be *Wolbachia*-free (Fernández-Ayala *et al.* 2010), strains BER1, Oregon R-C, CO3, Reids-1, and QI2 were found to be infected (Figure S1A). One of these stocks, BER1, was selected for detailed studies. By crossing infected BER1 females with tko^{25t} males, using the scheme shown in Figure S1B, we introgressed tko^{25t} into the BER1 cytoplasm (denoted wol; tko^{25t}). After confirming the presence of *Wolbachia* (Figure S1C, O'Neill *et al.* 1992), we tested the phenotype of the wol; tko^{25t} flies (Figure 1), finding a partial rescue of developmental delay (Figure 1A) and an almost complete suppression of bang sensitivity (Figure 1B). To test whether the presence of *Wolbachia* was the cause of this suppression, we treated wol; tko^{25t} flies with tetracycline over two generations, confirmed the elimination of the endosymbiont in the "cured" (Cwol; tko^{25t}) flies (Figure S1D), and then retested for phenotype. Surprisingly, we found an identical degree of suppression in the cured (Cwol; tko^{25t}) as in the infected wol; tko^{25t} flies (Figure 1, A and B), indicating that *Wolbachia* was not the suppressing determinant of the BER1 cytoplasm.

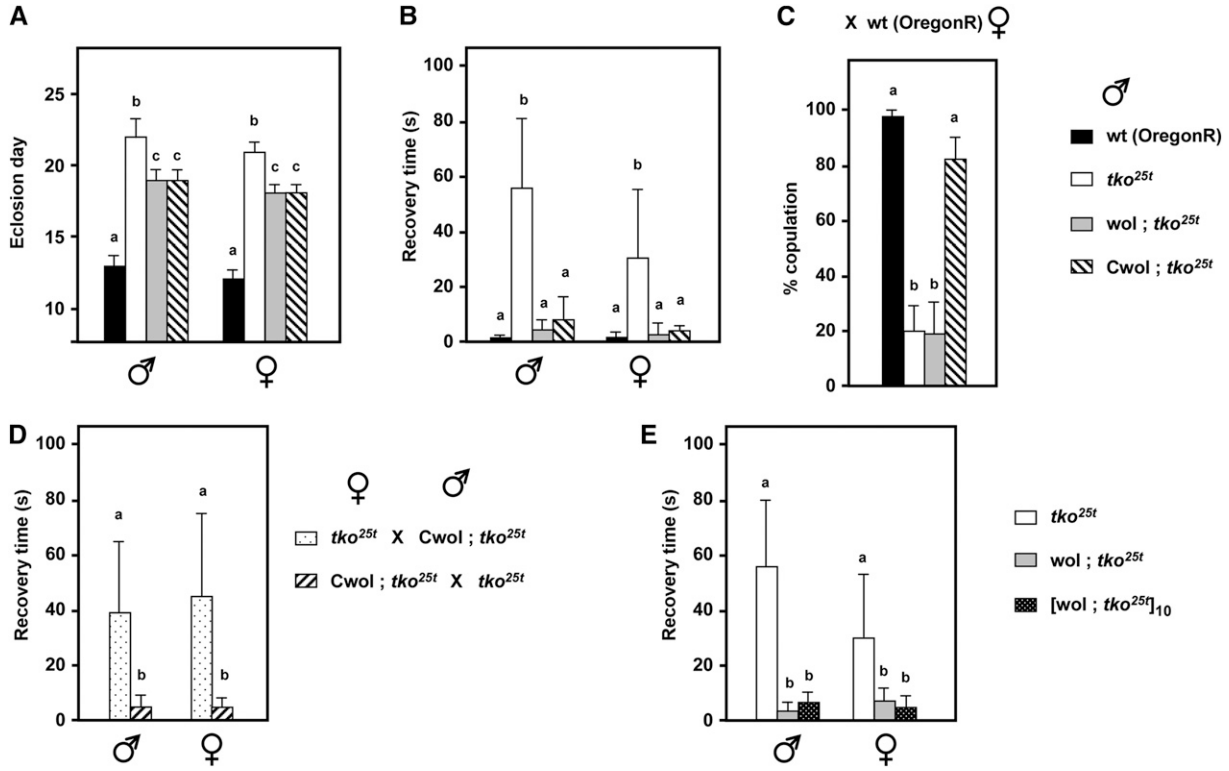

Figure 1 BER1 mtDNA confers partial suppression of the *tko*^{25t} phenotype. (A) Time to eclosion at room temperature and (B) bang sensitivity (recovery time from mechanical shock) of flies of the sex and genotypes indicated (means ± SD). wol, *Wolbachia*-infected BER1 cytoplasmic background; Cwol, BER1 cytoplasmic background after *Wolbachia* removal by tetracycline treatment, in each case crossed into *tko*^{25t} as shown in Figure S1B. (C) Frequency of successful copulation between wild-type (Oregon R) females and males of the indicated genotypes. (D) Bang sensitivity of progeny from reciprocal cross between *tko*^{25t} flies in the original and *Wolbachia*-cured (BER1) backgrounds. (E) Bang sensitivity of *tko*^{25t} flies in the *Wolbachia*-infected (BER1) background, before and after backcrossing for 10 generations to *tko*^{25t} males, alongside bang sensitivity of *tko*^{25t} flies in the original background, reproduced from A. A, B, and C denote significantly different data classes in each experiment (Newman–Keuls test, $P < 0.05$, based on ANOVA, $P < 0.001$, each sex considered separately).

To test the inheritance pattern of the suppressing determinant, we crossed Cwol; *tko*^{25t} females with *tko*^{25t} males from our original stock and *vice versa* (Figure S1E). The suppression of bang sensitivity was transmitted in a strictly maternal fashion (Figure 1D), indicating that it must reside in the mtDNA itself, or else in an unknown, maternally transmitted genetic element. To exclude any effects of nuclear genes, we backcrossed Cwol; *tko*^{25t} females to *tko*^{25t} males from the original strain in the Oregon R background over 10 generations, using the scheme of Figure S1F, and then retested the phenotype. The suppression of bang sensitivity was fully retained in the backcrossed flies (Figure 1E). The cured BER1 background also rescued the male courtship defect of *tko*^{25t}, although the corresponding *Wolbachia*-infected strain prior to curing was still defective for male courtship (Figure 1C). The original *Wolbachia*-infected wild-type strain was itself male-courtship defective, when tested in this assay with Oregon R females (giving a copulation frequency of only 57%). Finally, the BER1-derived mtDNA slightly increased the lifespan of *tko*^{25t} males, but not females (Figure S1G), after homogenization of the nuclear background by backcrossing.

To gain insight into the mechanism of suppression, we assayed total ATP levels in homogenates of adult flies of the different strains and measured mtDNA copy number by qPCR. Both parameters showed a correlation with phenotypic suppression. The total ATP level in the original *tko*^{25t} strain was significantly lower than in the wild-type Oregon R reference strain, but was restored to almost the wild-type level in wol; *tko*^{25t} or Cwol; *tko*^{25t} flies (Figure 2A). Normalized to mitochondrial protein concentration, respiration by isolated mitochondria was much lower in *tko*^{25t} adults than in controls (Figure 2B), while mitochondrial ROS production was increased (Figure 2C). Neither of these parameters was significantly altered by the BER1 mitochondrial background (Cwol; *tko*^{25t}). We conclude that BER1 mtDNA does not alter the biochemical phenotype as such. Therefore, we next tested whether it might act simply by increasing the amount of mtDNA and of mitochondria.

The copy number of mtDNA was significantly higher in wol; *tko*^{25t} or Cwol; *tko*^{25t} than in *tko*^{25t} in the original (Oregon R) background, with the differences more pronounced in males (Figure 2D). There was also a substantial recovery in the ratio of small subunit to large subunit mitoribosomal RNA (Figure 2E, Figure S2D). Furthermore, as measured by the levels of the outer mitochondrial membrane marker protein porin and of the nuclear-coded

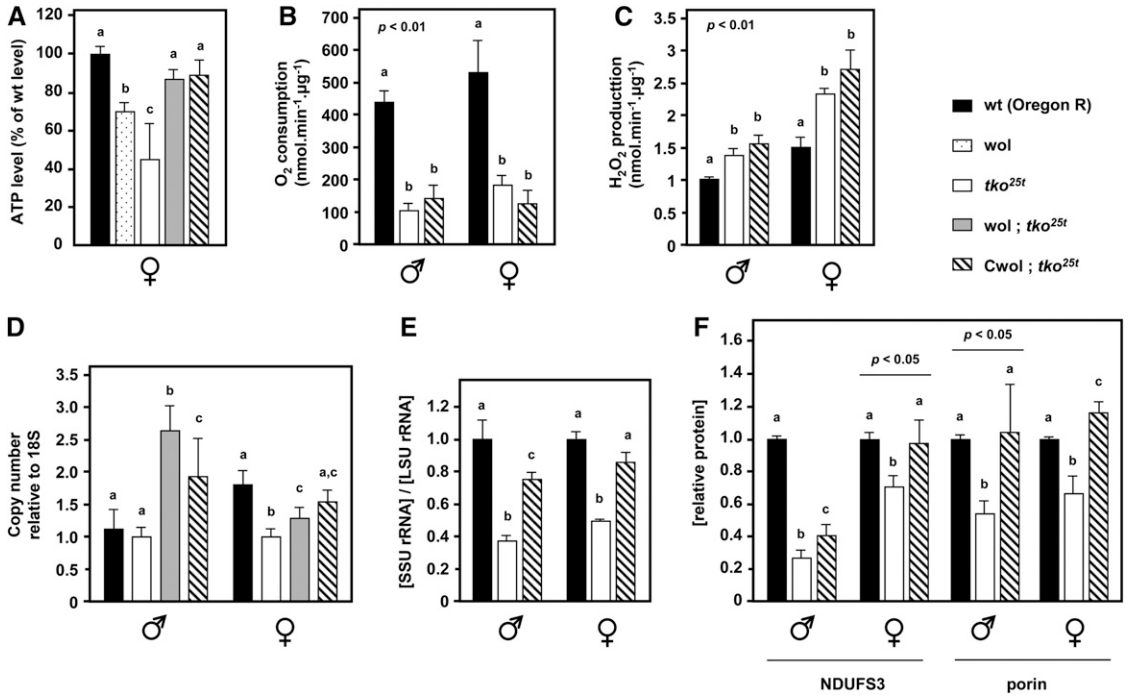

Figure 2 BER1 mtDNA influences ATP, mtDNA, mtRNA, and protein levels in *tko*^{25t} flies. (A) ATP levels in whole flies (mean ± SD), plus (B) oxygen consumption, and (C) ROS production of isolated mitochondria from flies of the indicated sex and genotypes, mean ± SE, three or more biological replicates. (D) Copy number of mtDNA in flies of the genotypes indicated, relative to 18S rDNA, means ± SD, normalized to that in *tko*^{25t} flies of the same sex. (E) Ratio of mitoribosomal SSU to LSU RNA levels in flies of the indicated genotypes, means ± SD wol; *tko*^{25t} and Cwol; *tko*^{25t} flies, maintained as balanced stocks, were backcrossed over five or more generations to *tko*^{25t} in the Oregon R background and rendered homozygous prior to the experiment. (F) Levels of a representative subunit of complex I (NDUFS3) and of a global mitochondrial protein marker (porin) in flies of the sex and genotype indicated, based on densitometry of Western blots as shown in Figure S2E, normalized against a cytosolic loading control (α-actinin). A, B, and C denote significantly different data classes (Newman–Keuls test, $P < 0.05$, based on ANOVA, $P < 0.001$ except where indicated, each sex and, where appropriate, each gene considered separately).

complex I subunit NDUFS3 (Figure 2F, Figure S2E), relative to a cytosolic marker, there was a significant increase in mitochondrial protein from the depressed levels seen in the original *tko*^{25t} line, although the increase in some other mitochondrial proteins, such as ATP synthase subunit α, was much more modest (Figure S2F), though still significant. Importantly, mtDNA copy number was not elevated in otherwise wild-type flies carrying the BER1 cytoplasm (Figure S2C).

Mitochondrially inherited suppression of a mitochondrial protein synthesis defect by a mechanism that generates additional capacity for ATP synthesis logically should not affect mutants with defects in ATP supply downstream of OXPHOS, such as *sesB*^{1} (encoding the major isoform of the adenine nucleotide translocase, Zhang *et al.* 1999). To test this, we crossed the *Wolbachia*-containing cytoplasm from BER1 into *sesB*^{1}, using the same strategy as for *tko*^{25t} (Figure S1D), and then tested the phenotype. In this case there was no rescue of bang sensitivity (Figure 3A), developmental delay (Figure 3B), or female sterility. There was, however, a modest rescue of the short-lifespan phenotype of *sesB*^{1} (Figure S3).

Next we tested the four other *Wolbachia*-infected strains for suppression of *tko*^{25t}, using a similar crossing strategy as for BER1, with at least five generations of backcrossing in each case. Two strains (CO3 and Reids-1) were phenotypically tested in the uncured state, whereas the two others (QI2 and Oregon R-C) were tested after curing by tetracycline treatment. The cytoplasm from backgrounds QI2, Reids-1, and CO3 gave a similar degree of rescue of the mutant phenotype as did that of BER1 (Figure 4, A and B), while Oregon R-C cytoplasm gave no rescue. A control crossing strategy using wild-type Oregon R as the cytoplasmic donor also produced no rescue, nor did cytoplasm from wild-type strain Canton-S (Figure S4A). When tested, the nuclear background of all these strains was the same, further supporting the view that the suppression activity is entirely cytoplasmic. Importantly, all four suppressor cytoplasms (after removal of *Wolbachia*) showed elevated mtDNA copy number in the *tko*^{25t} background, whereas nonsuppressor Oregon R-C cytoplasm did not (Figure 4C).

Logically, a cytoplasmic suppressor acting via an effect on the amount of mtDNA should reside in portions of the mitochondrial genome affecting replication or copy number control. However, the *cis*-acting signals regulating mtDNA copy number remain to be identified. Moreover, such an effect might also be indirect, *e.g.*, mediated by the properties of the mitochondrially encoded gene products themselves. We therefore sequenced the entire coding region of the mtDNA of the four suppressor strains, plus three nonsuppressor strains

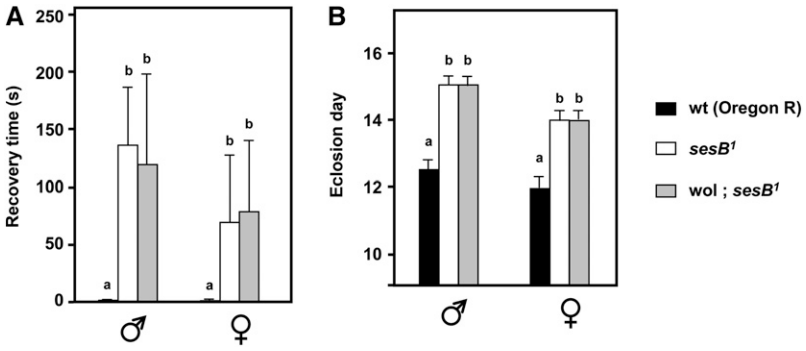

Figure 3 BER1 strain mtDNA does not rescue the developmental phenotype of *sesB*^{1}. (A) Bang sensitivity (mean recovery times ± SD) and (B) time to eclosion at room temperature of flies of the sex and genotypes indicated. wol, *Wolbachia*-infected BER1 cytoplasmic background, crossed into *sesB*^{1}. A and B denote significantly different data classes (Newman–Keuls test, $P < 0.05$, based on ANOVA, $P < 0.01$, each sex considered separately).

(Canton-S, Oregon R-C, and the mtDNA of the original *tko*^{25t} strain; the coding region of Oregon R is already deposited in the NCBI database, accession no. AF200828). We also characterized the structure and determined the sequence of the NCR (A + T rich and highly repetitive) in representative strains.

The mtDNA coding regions of all strains analyzed contained between 30 and 50 single nucleotide or short indel polymorphisms compared with the reference sequence, NCBI NC_001709 (Table S1). Each strain had a unique sequence, although suppressor strains CO3 and QI2 were very similar, as were nonsuppressor strain Oregon R-C (identical with the previously sequenced Oregon R strain) and the original *tko*^{25t} stock, which differed by only two polymorphisms. Suppressor strain BER1 and nonsuppressor strain Canton-S shared many polymorphisms not found in other strains. No single coding-region polymorphism was present in all suppressor strains but absent from all nonsuppressor strains (or *vice versa*), nor was any significant heteroplasmy detected.

The overall structure of the NCR was inferred for several suppressor and nonsuppressor strains using a combination of long PCR (Figure S4B), partial restriction mapping (Figure S4, C–E), and Sanger sequencing, involving both PCR and cloning (Figure S4, F and G). The NCR of BER1 mtDNA, as well as that of most other strains, including both suppressors and nonsuppressors, was the "short" morph, containing only three copies of repeat I, whereas the reference sequence (Lewis *et al.* 1994) is the "long" morph, containing five (Figure S4H). Complete NCR sequences of suppressor strain BER1 and the original (nonsuppressor) *tko*^{25t} strain revealed many single-nucleotide and short indel polymorphisms distinguishing the NCRs of these strains from each other and from the reference sequence, many of them affecting the length of oligo(A/T) and oligo(AT/TA) tracts, but also some rare AT-to-GC transitions. There was no evidence in the NCR of BER1 mtDNA for the presence of integrated sequences derived from *Wolbachia*, nor was there any obvious or systematic difference between the NCRs that might explain the suppressor phenotype.

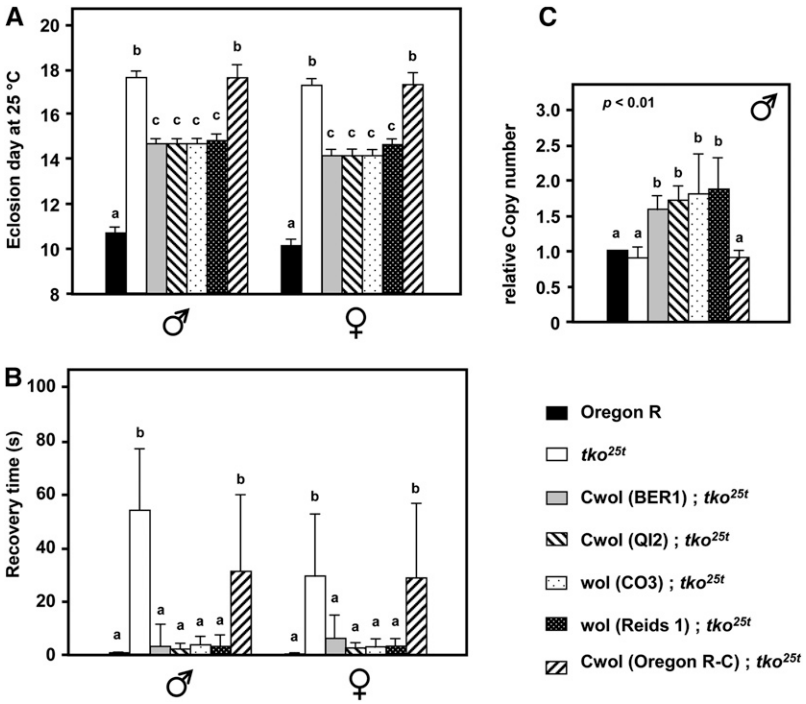

Figure 4 mtDNA from three other *Wolbachia*-infected backgrounds partially suppresses *tko*^{25t} phenotype. (A) Time to eclosion at 25°, (B) bang sensitivity, and (C) mtDNA copy number of flies of the sex and genotypes indicated. wol, strains still infected with *Wolbachia*; Cwol, strains cured of *Wolbachia* infection. 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).

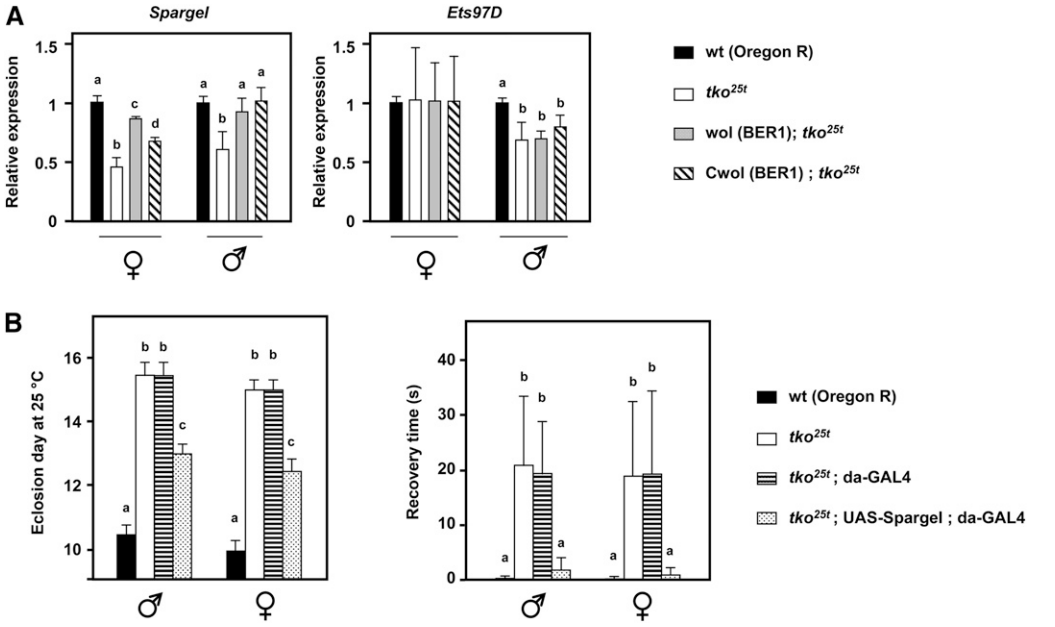

Figure 5 Expression levels of global regulators of mitochondrial biogenesis in *tko*^{25t} in different mtDNA backgrounds. (A) qRT–PCR of *Spargel* and *Ets97D* mRNAs normalized to that of *RpL32* and then to the values for wild-type Oregon R flies of the given sex and genotype. a, b, c, and d denote significantly different data classes (Newman–Keuls test, $P < 0.05$, based on ANOVA, $P < 0.01$, each gene in each sex considered separately). Despite the variation between the sexes, and between flies with and without the physical presence of *Wolbachia*, both the downregulation of *Spargel* in *tko*^{25t} flies and its restoration in the presence of the BER1 cytoplasm are statistically significant. (B) Bang sensitivity (mean recovery times ± SD) and time to eclosion at 25 ° of flies of the sex and genotypes indicated. For the crosses required in this experiment, the *UAS-Spargel* transgene was first rendered homozygous in the *tko*^{25t} background, with the original nonsuppressor cytoplasm. a, b, and c denote significantly different data classes (Newman–Keuls test, $P < 0.05$, based on ANOVA, $P < 0.01$).

We investigated the expression levels of eight genes with known or hypothesized roles in mtDNA replication or transcription, using qRT–PCR, revealing no significant differences between *tko*^{25t} in the original or *Wolbachia*-cured BER1 (Cwol; *tko*^{25t}) backgrounds (Figure S5A). Next (Figure 5) we investigated two genes proposed to have general roles in the regulation of mitochondrial biogenesis, namely *Spargel*, homolog of human *PPRC*, and *PPARGC1A* (PGC1α), and *Ets97D*, also known as *Delg*, ortholog of human *GABPA* (NRF-2α). *Ets97D* was slightly downregulated in *tko*^{25t} males, but was not significantly altered by the mtDNA background in either sex (Figure 5B). However, *Spargel* was significantly downregulated in *tko*^{25t} in both sexes, but restored to near wild-type expression in the BER1 mtDNA background, regardless of the presence or absence of *Wolbachia* (Figure 5A). This is consistent with the BER1 cytoplasm suppressing the phenotype of *tko*^{25t} by promoting mitochondrial biogenesis and suggests *Spargel* could be a key target in this process. To confirm this, we drove *Spargel* overexpression throughout development in *tko*^{25t} flies (Figure S5B), using the strong and ubiquitous da-GAL4 driver and a *UAS-Spargel* transgenic line (Tiefenböck *et al.* 2010), which together phenocopied the suppressor (Figure 5B), both for bang sensitivity and eclosion timing.

Discussion

Nature of the tko^{25t} *suppressor*

In this study, we set out to test the role of cytoplasmic genetic determinants on the phenotype of *tko*^{25t}, a *Drosophila* nuclear OXPHOS mutant. Cytoplasm from the *Wolbachia*-infected strain BER1 partially suppressed *tko*^{25t}, rescuing bang sensitivity and male courtship defect, and alleviating developmental delay. Based on reciprocal crosses and backcrossing over 10 generations, the suppressor determinant was entirely cytoplasmic, but independent of the physical presence of *Wolbachia*, because the tetracycline-cured BER1 strain retained full suppression. The cytoplasmic determinant providing suppression most likely resides in mtDNA itself, although we cannot completely exclude some other maternally inherited element that has escaped detection, such as a tetracycline-resistant, prokaryotic endosymbiont not detected by PCR using universal primers, or a cytoplasmically transmitted plasmid or virus. Suppression was associated with increased mitochondrial biogenesis and mtDNA copy number, supporting the idea that the suppressor resides in mtDNA, *e.g.*, as a *cis*-acting element that responds to mitochondrial stress. The most logical location for such an element would be within the NCR, which contains the replication origin and terminus (Goddard and Wolstenholme 1978; Saito *et al.* 2005). At this time, nothing is known of the physiological mechanisms of mtDNA copy-number control in any metazoan, although many genes are known to be required for mtDNA maintenance (Larsson *et al.* 1998; Iyengar *et al.* 1999, 2002; Matsushima *et al.* 2004; Hance *et al.* 2005).

Natural length variation in the NCR (Townsend and Rand 2004) has been suggested to have selective value, with longer NCR variants hypothesized as advantageous (Solignac *et al.* 1987; Kann *et al.* 1998). However, the overall architecture of the NCR did not correlate with suppressor activity. Nonsuppressor strain Canton-S had a long NCR equivalent to that of the reference sequence, with five copies of repeat I, whereas all other strains tested had a shorter NCR (three copies of

repeat I, Figure S4), regardless of whether they were suppressors or nonsuppressors.

The NCR contains many strain-specific and shared polymorphisms, notably in the length of oligo(A/T) and oligo (AT/TA) tracts. Suppressor strain BER1 had ~80 such polymorphisms compared with the reference sequence or the original *tko*^{25t} strain, including some transitions at phylogenetically conserved positions (Brehm *et al.* 2001). However, large-scale sequencing and analysis of many wild strains will be needed to identify any specific NCR polymorphism(s) correlating with suppressor activity.

Coding region polymorphisms may also underlie haplotype-specific copy-number regulation (Suissa *et al.* 2009), for example, by influencing the binding of specific proteins. However, no single polymorphism distinguished suppressor from nonsuppressor strains, nor did we find any evidence for heteroplasmy. Globally, suppressor strain BER1 was most similar to nonsuppressor strain Canton-S, whereas suppressor strains QI2, CO3, and Reids-1 were more similar to nonsuppressor strains Oregon R and Oregon R-C (Table S1). The suppressor thus appears to have arisen independently on different haplotype backgrounds.

The recent confirmation of the presence of both cytosine methylation and hydroxymethylation in mammalian mtDNA (Shock *et al.* 2011) suggests the possibility that the suppressor might be an epigenetic mark carried in the mitochondrial genome rather than the mtDNA sequence itself. The increased expression of the mitochondrial isoform of the DNA methyltransferase DNMT1 in human cells under hypoxia (Shock *et al.* 2011) is accompanied by altered mitochondrial gene expression, raising the possibility that an epigenetic alteration of mtDNA might underlie the resistance to the stress imposed by the *tko*^{25t} mutation.

Mechanism of the mitochondrial suppressor of tko^{25t}

The degree of phenotypic rescue was similar in four different suppressor strains, indicating a common mechanism. Although respiration and ROS production were unchanged, when normalized against the amount of mitochondrial protein, the amount of mitochondrial protein was itself increased up to twofold, accompanied by an increased mtDNA copy number and a shift in the ratio SSU:LSU rRNA toward the equimolarity typical of wild-type flies, as seen also in a nuclear suppressor (Kemppainen *et al.* 2009), and indicating enhanced mitochondrial biogenesis as the key mechanism of suppression.

The suppressor was independent of any short-term copy-number increase after tetracycline treatment (Ballard and Melvin 2007), because it persisted for many generations. There was also increased expression of *Spargel* (PGC1α), one of the proposed global regulators of mitochondrial biogenesis and retrograde signaling (Spiegelman 2007; Jones *et al.* 2012). Importantly, *Spargel* expression was found to be low in *tko*^{25t} flies in the nonsuppressor background, and simply restored to wild-type levels in the presence of the suppressor. This may indicate a programmed downregulation of mitochondrial biogenesis in nonsuppressor backgrounds in response to the metabolic stress generated by *tko*^{25t} (Fernández-Ayala *et al.* 2010). Suppressor mtDNAs might be refractory to mitochondrial stress signals, or else the gene products of suppressor mtDNA may minimize the production of these signals. Note that the copy number of suppressor-strain mtDNA was not elevated in a wild-type nuclear background, consistent with the idea that the (sex-specific) changes seen in *tko*^{25t} are a stress response rather than an invariant property of the suppressor-strain mtDNAs. Crucially, ubiquitous overexpression of *Spargel* was able to phenocopy the suppressor, showing that the expression level of *Spargel* is not merely a marker for the effect of the suppressor, but is a key mechanistic target thereof.

The signal to which suppressor- and nonsuppressor-strain mtDNAs may respond differently could be a direct metabolic readout of OXPHOS insufficiency or an indirect effect of defective mitochondrial translation, such as a disturbance in protein homeostasis (Dieteren *et al.* 2011). Different mtDNA backgrounds proposed as modifiers of Leber Hereditary Optic Neuropathy in humans have been previously shown to modulate OXPHOS complex assembly (Pello *et al.* 2008). In *tko*^{25t}, one of the most highly upregulated genes is *Hsp22* (Fernández-Ayala *et al.* 2010), also upregulated under stress and in aging (Morrow *et al.* 2004; Yang and Tower 2009; Kim *et al.* 2010). Suppressor- and nonsuppressor-strain mtDNAs may thus differ in their induction or implementation of the mitochondrial unfolded protein response (Haynes and Ron 2010).

The suppressor clearly does not act by establishing a bypass of ATP deficiency, since the developmental phenotype of *sesB*^{1}, affecting the mitochondrial adenine nucleotide translocator ANT (Zhang *et al.* 1999), was not rescued by BER1 cytoplasm. However, the adult phenotype of *sesB*^{1}, similar to that of the cytochrome *c* oxidase mutant *levy*^{1} (Liu *et al.* 2007) was partially alleviated. The phenotype may reflect enhanced oxidative stress and damage, against which the suppressor mtDNA affords some protection. Some molecular correlates of suppression, including *Spargel* expression, mtDNA copy number, and the amount of assembled complex I, as judged by the NDUFS3 marker, exhibited quantitative differences between the sexes, even though all markers showed significant improvement in both males and females in the presence of suppressor cytoplasm. Sex differences have been reported previously in mitochondrial bioenergetics and oxidative stress handling (Ballard *et al.* 2007b), and these might underlie the differences observed here. Isolation and study of additional mutants with compromized OXPHOS and/or defects in mitochondrial biogenesis should help to elucidate further the relationship between mitochondrial stress signaling, mtDNA copy number, and the suppressor determinant.

Role of Wolbachia in selecting suppressor cytoplasms

Cytoplasm from four wild strains of *Drosophila* naturally infected with *Wolbachia* conferred suppression after curing

of *Wolbachia*, while that from two *Wolbachia*-free wild-type strains (Oregon R and Canton-S) did not. However, the correlation between *Wolbachia* infection and suppressor activity is not absolute, since *Wolbachia*-infected strain Oregon R-C, collected from the wild at the same time as the closely related strain Oregon R (Table S1), was also a nonsuppressor. Oregon R-C was first reported as infected in 1994 (Bourtzis *et al.* 1994), but has been maintained in laboratories for over 50 years (Clancy and Beadle 1937), and we cannot exclude the possibility that it became infected in the laboratory. Suppressor activity in the wild may therefore have been selected under the metabolic stress imposed by *Wolbachia*.

Wolbachia is considered an endosymbiont, with variable effects on host reproduction to favor its own spread in the population (Stouthamer *et al.* 1999; Champion de Crespigny *et al.* 2006), although host adaptation may also subvert this process (Turelli 1994). Effects on host fitness are influenced dramatically by mitochondrial genetic background in *D. simulans* (Dean 2006), since *Wolbachia* may compete with mitochondria for substrates. Flies of a genetic background able to boost mitochondrial functions in the presence of such an intruder might be expected to have a natural advantage.

Paradigm for understanding nuclear–mitochondrial interactions in human disease

Suppressor and nonsuppressor cytoplasmic backgrounds were isolated in multiple examples from nature, and presumably have a different adaptive value depending on the conditions, as hypothesized for different mtDNA haplotypes in the human population (Wallace 2005). In a wild-type nuclear background, suppressor and nonsuppressor cytoplasms confer no obvious biological advantage or disadvantage, but in the tko^{25t} background, where mitochondrial protein synthesis is limiting, the effects are substantial.

Many human pathologies result directly from defects in mitochondrial protein synthesis (Jacobs and Turnbull 2005; Rötig 2011), including mutations in genes for mitoribosomal proteins (Miller *et al.* 2004; Saada *et al.* 2007; Smits *et al.* 2011) and numerous other translation factors. Our findings support the concept that the variable clinical phenotypes seen in these disorders reflect differences in mtDNA genotype.

The role of mtDNA copy number as a modifying factor in mitochondrial disease has long been hypothesized (Tyynismaa and Suomalainen 2009) and is supported by cell culture studies (Bentlage and Attardi 1996). Copy-number aberrations are features of some mtDNA pathologies and may contribute to the clinical phenotype (Liu *et al.* 2006; Brinckmann *et al.* 2010), in addition to those disease entities where mtDNA maintenance is a primary pathological target (Lamperti and Zeviani 2009; Rötig and Poulton 2009). They have also been reported in other diseases, such as breast cancer (Shen *et al.* 2009).

Artificial manipulation of mtDNA copy number can influence disease phenotype in animal models (Ekstrand *et al.* 2004; Matsushima *et al.* 2004; Tyynismaa *et al.* 2004; Ikeuchi *et al.* 2005; Hokari *et al.* 2010; Matsushima *et al.* 2010; Nishiyama *et al.* 2010), although high copy number is not always beneficial (Ylikallio *et al.* 2010).

Although we remain ignorant of the physiological mechanisms regulating mtDNA copy number and its responsiveness to stress, the tko^{25t} suppressor provides a paradigm for mtDNA sequence variation playing a role as a disease modifier in humans. The further exploitation of this model and elucidation of the underlying molecular machinery may reveal new drug targets in what is currently an intractable class of human disease.

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A Cytoplasmic Suppressor of a Nuclear Mutation Affecting Mitochondrial Functions in *Drosophila*

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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

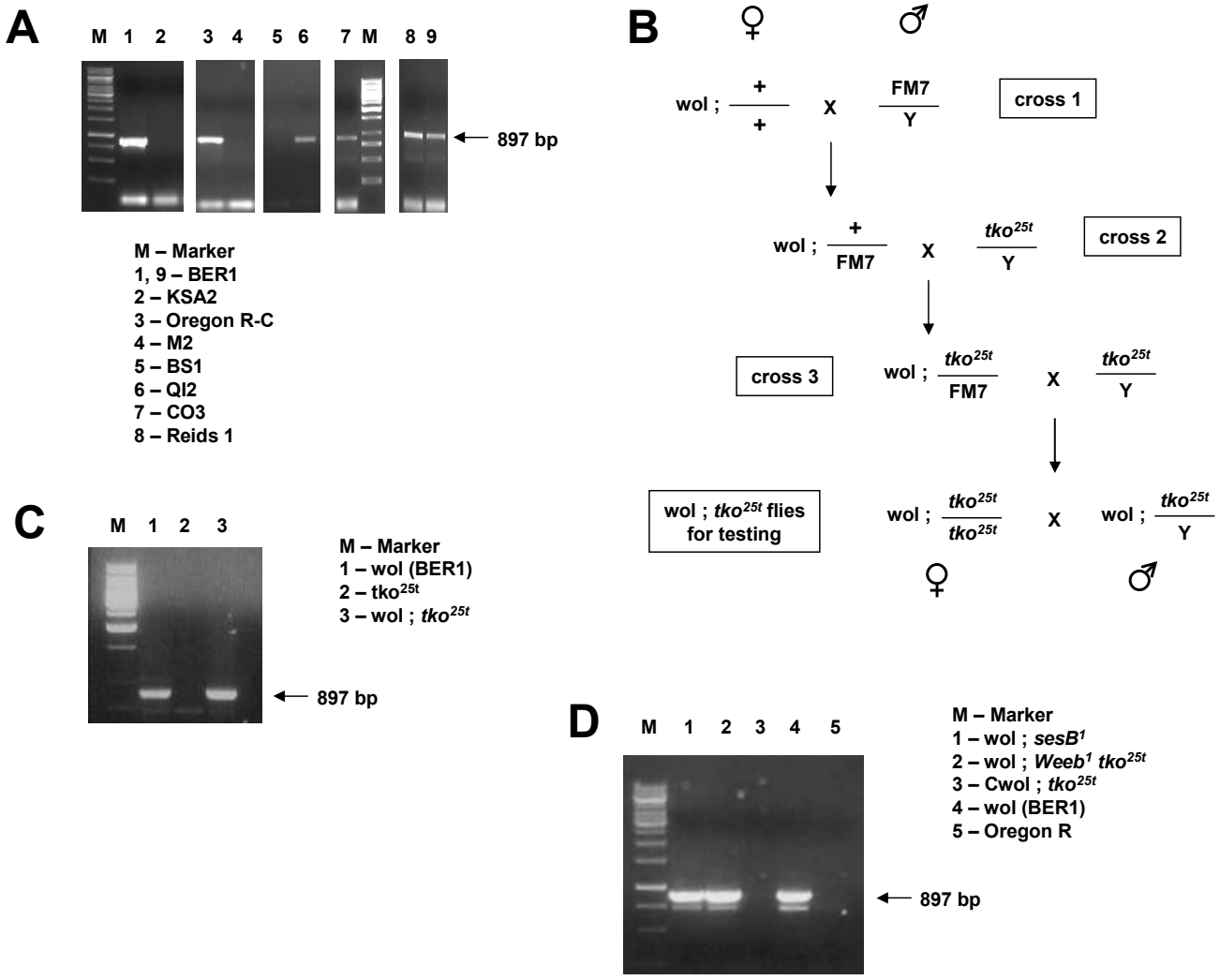
enzyme and 3 µl (30 µCi) of labeled ATP. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1 µl of 0.5 M EDTA (pH 8.0) and further incubation at 75 °C for 10 min. The unincorporated label was removed by gel filtration on Sephadex G-50 (GE healthcare). The mtDNA noncoding 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

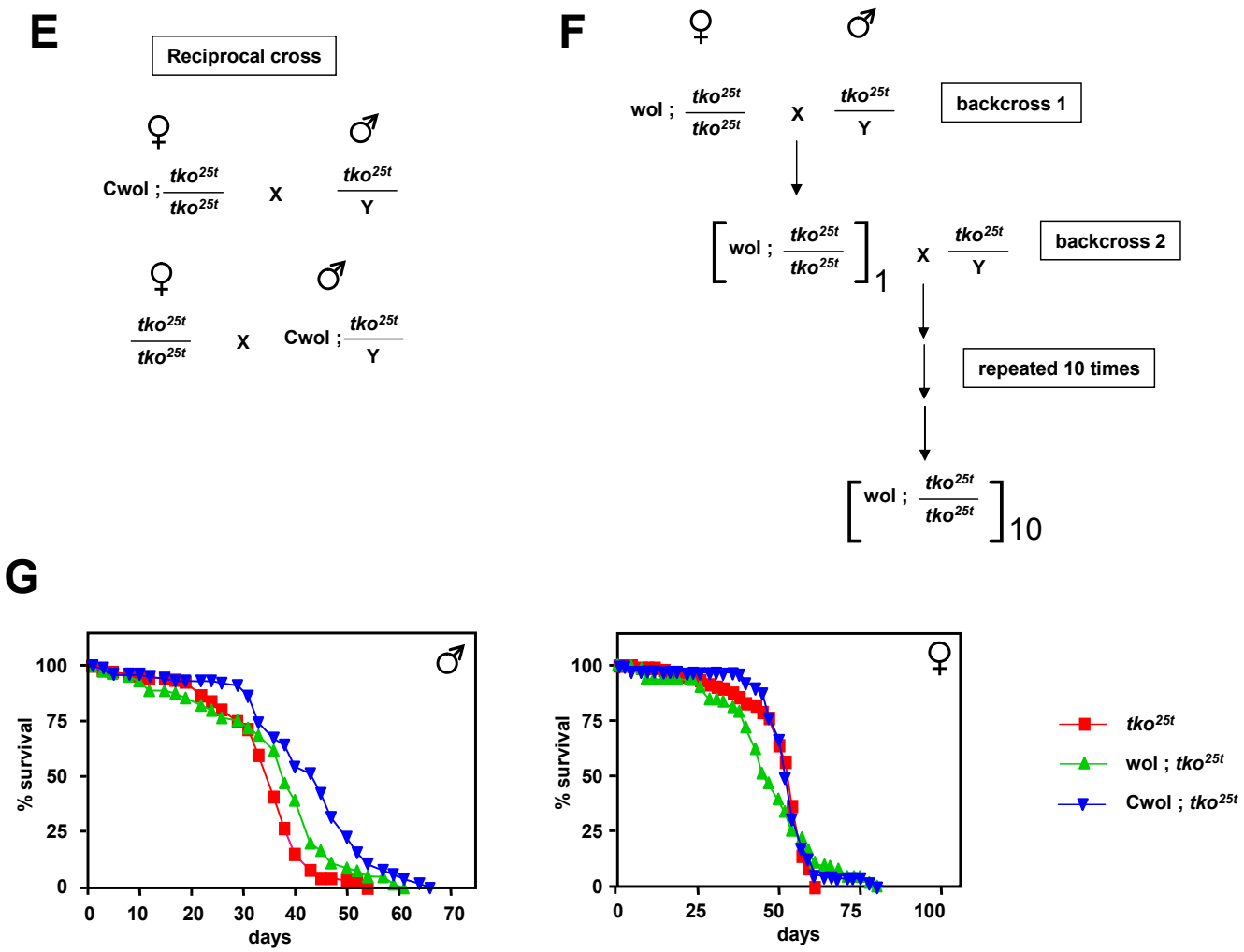
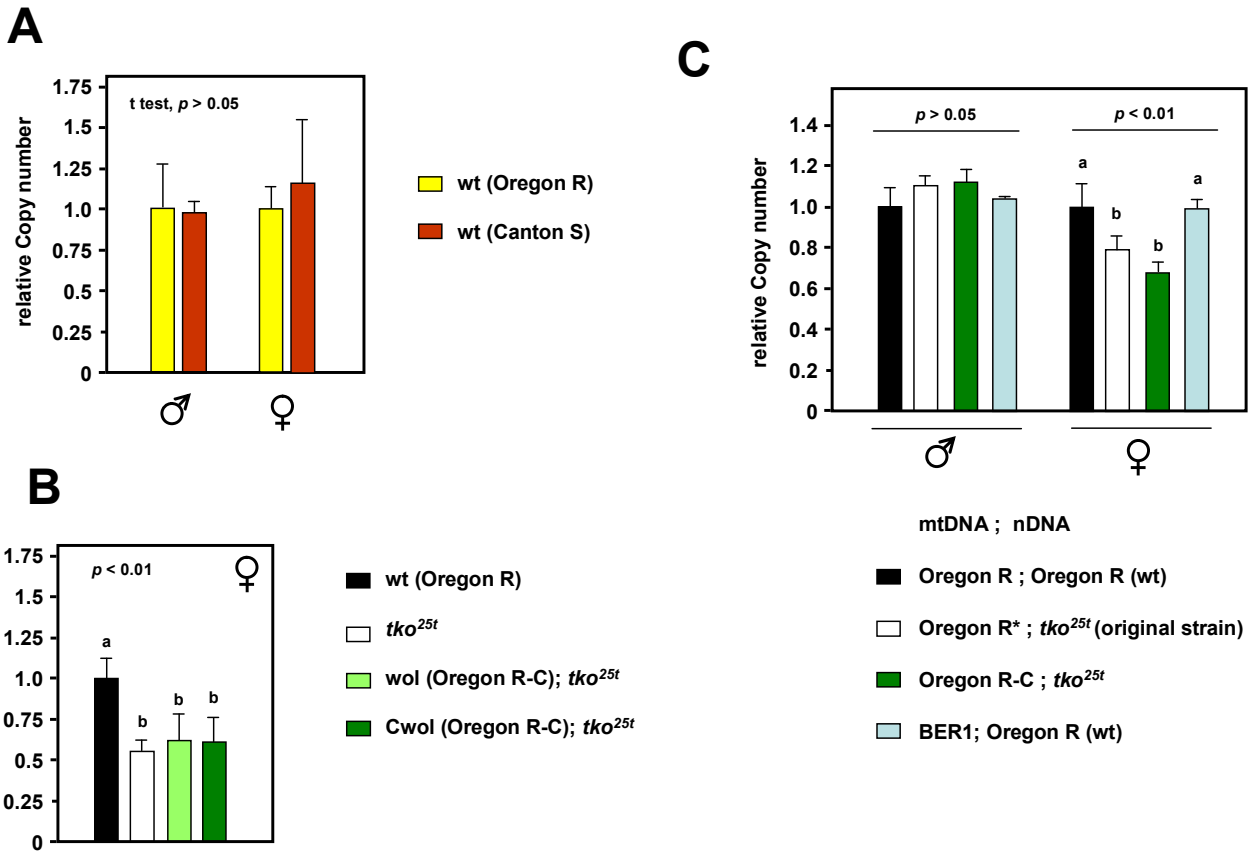


Figure S1 Creation and phenotypic characterization of cybrid *tko*^{25t} and *sesB*^{1} flies. (A) Representative gels of PCR products obtained using the *Wolbachia* 16S rRNA gene primers on genomic DNA of single individuals from the wild-type strains indicated. Gels run in parallel using the same marker 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)

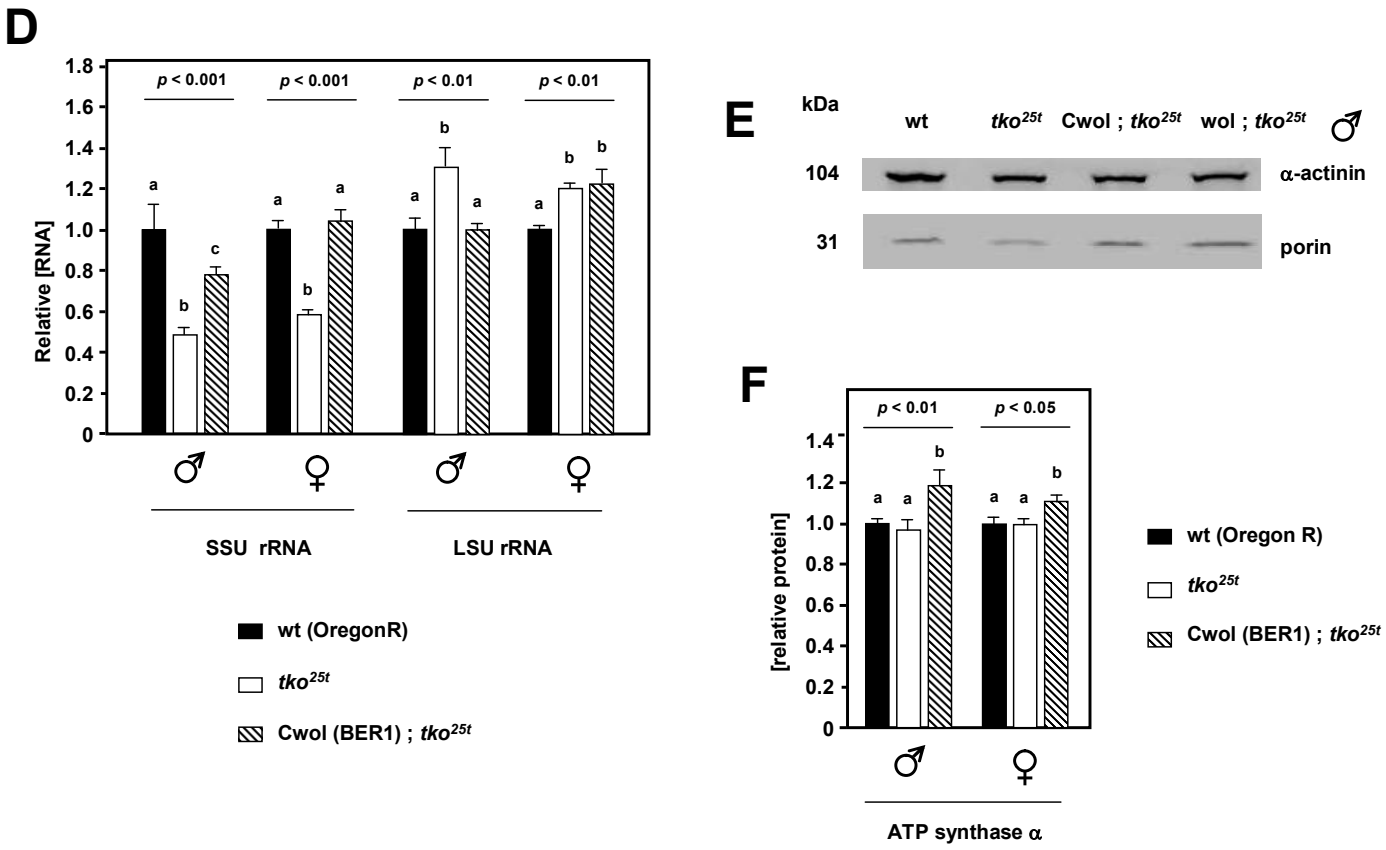


Figure S2 Molecular correlates of partial suppression of *tko*^{25t} by the suppressor mtDNA backgrounds. (A) Supplementary data to Fig. 2D: copy number of mtDNA in two different wild-type strains is similar. Copy number, relative to 18S rDNA, means ± SD, was normalized to that in Oregon R flies of the 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).

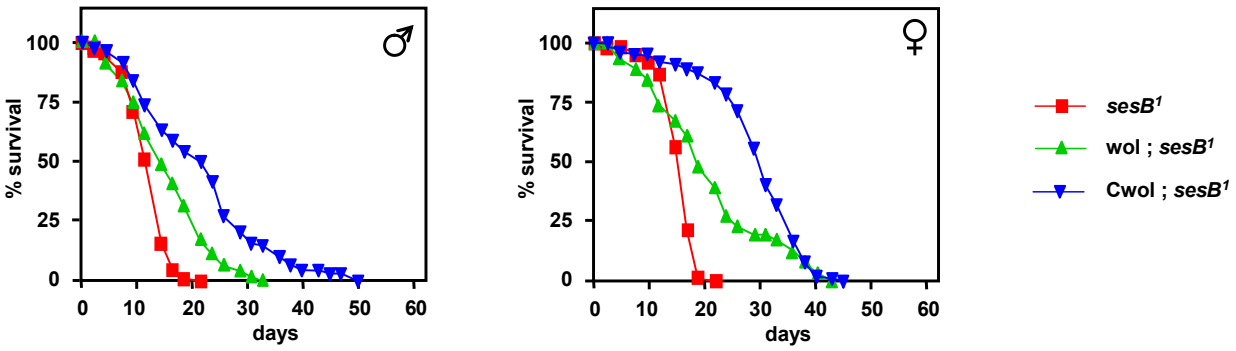
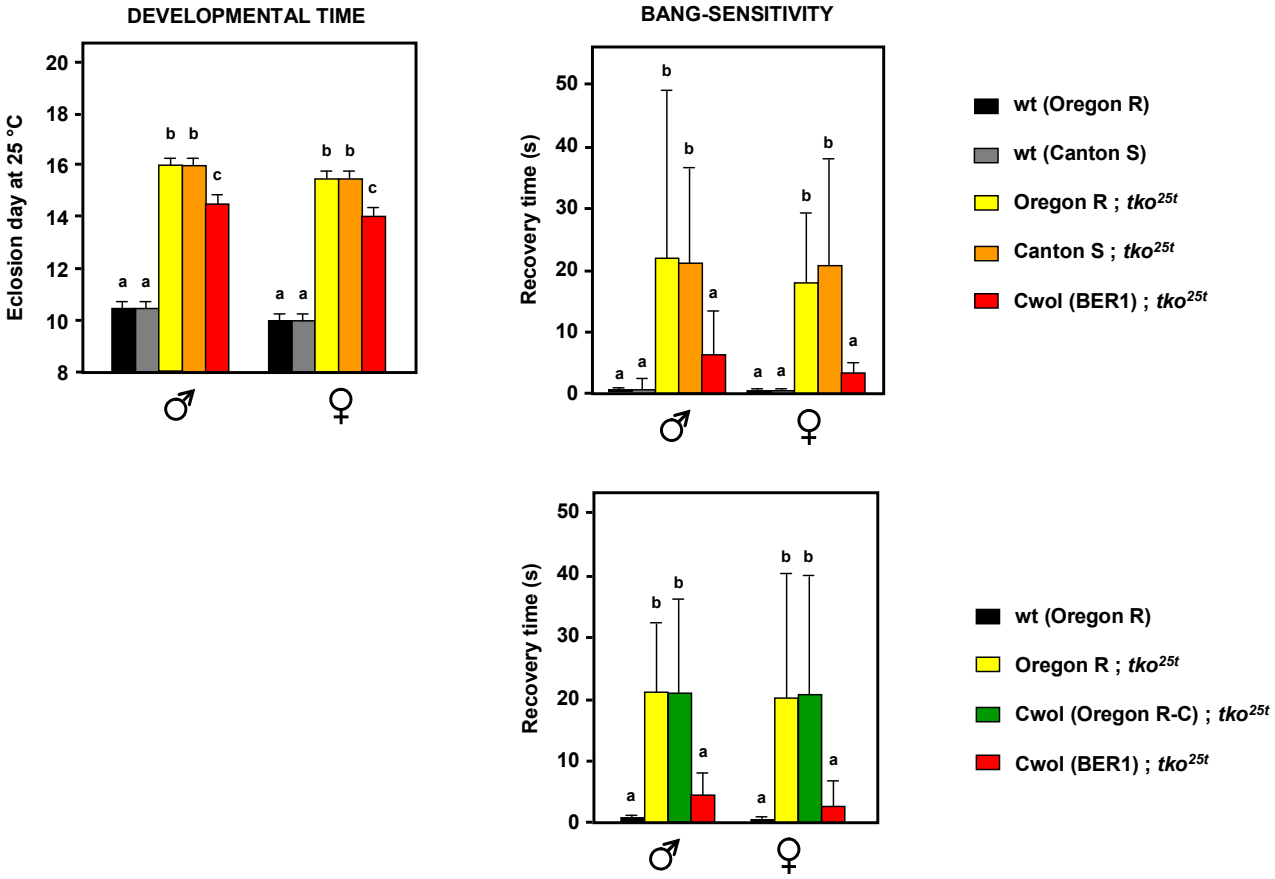
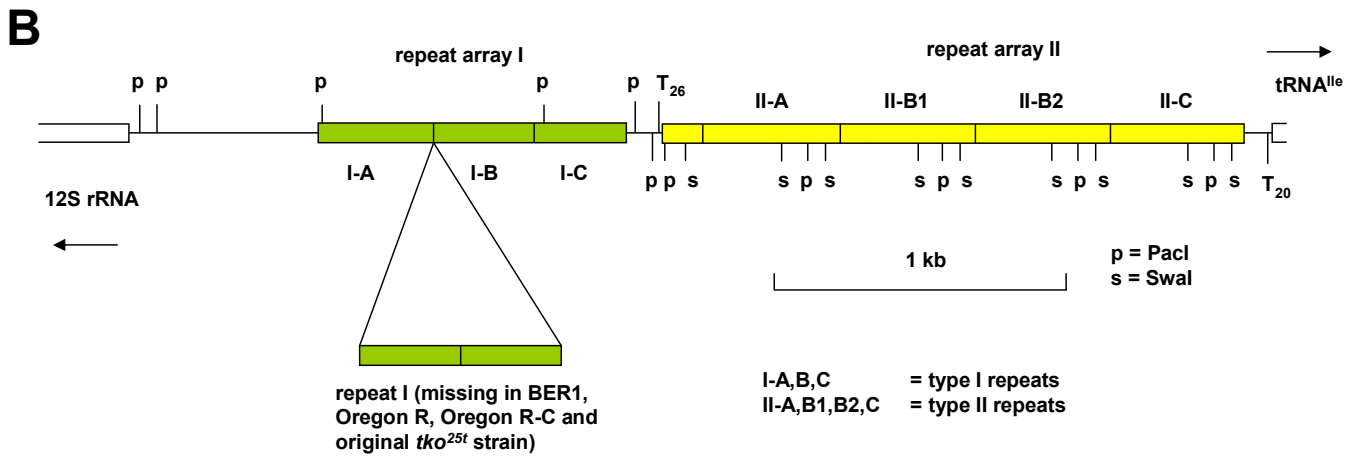
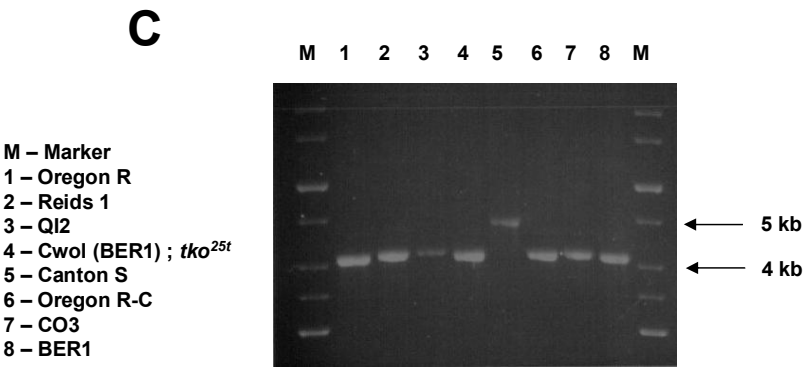


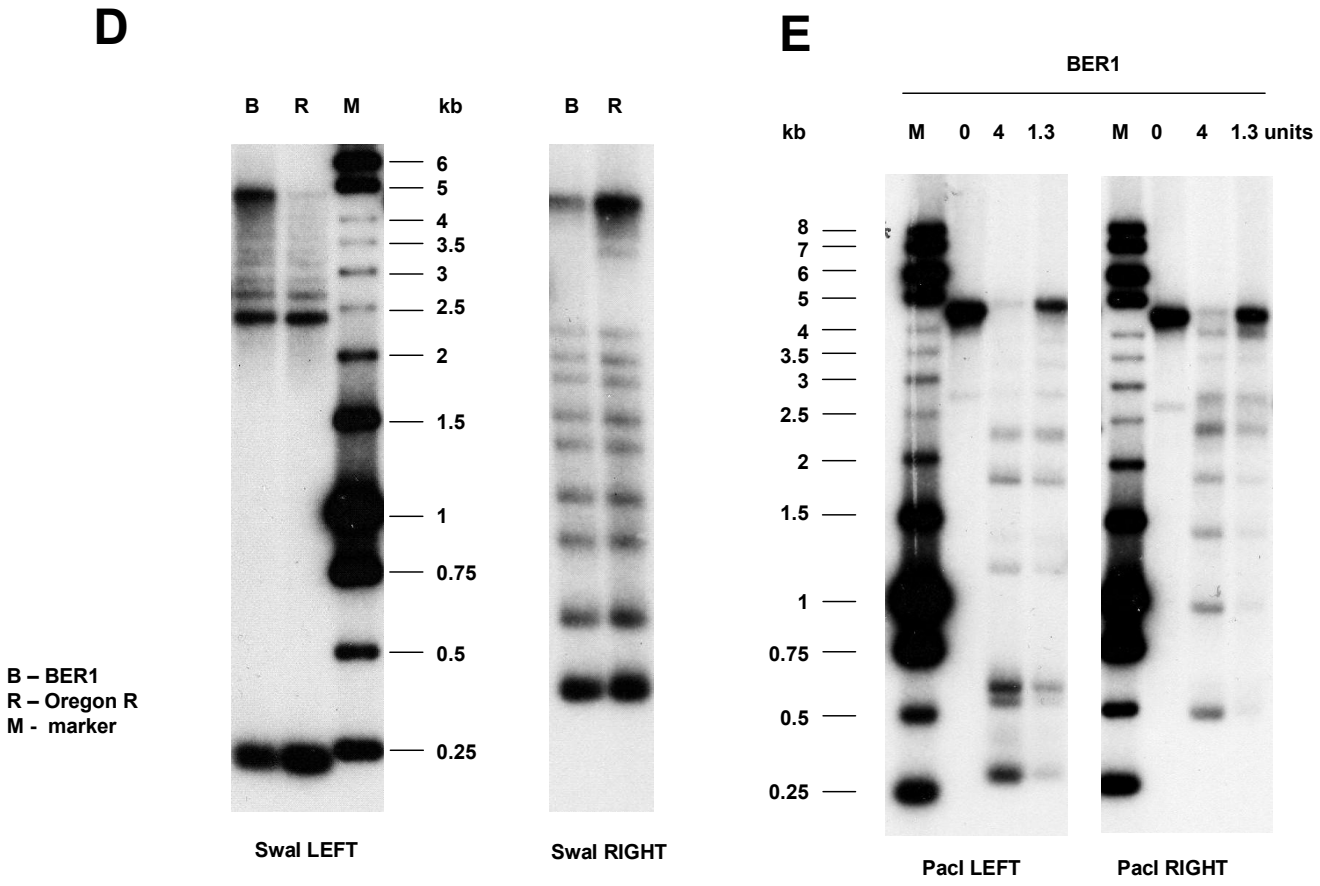
Figure S3 BER1 strain mtDNA increases the lifespan of *sesB*^{1} flies. Lifespan curves for flies of the genotypes indicated. For both sexes, the curves for wol (BER1); *sesB*^{1} and Cwol (BER1) ; *sesB*^{1} (median survival of 17, 19 d, respectively, for males, 31 and 26 d for females) were significantly 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

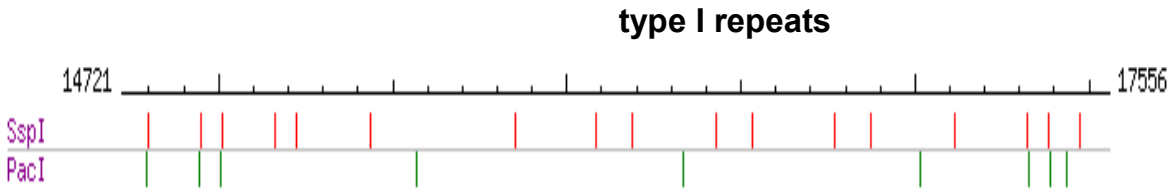

DEVELOPMENTAL TIME
Eclosion day at 25 °C
20
18
16
14
12
10
8
a a
b b
c
a a
b b
c
♂
♀
BANG-SENSITIVITY
Recovery time (s)
50
40
30
20
10
0
a a
b
b
a
a a
b
b
a
♂
♀
wt (Oregon R)
wt (Canton S)
Oregon R ; tko25t
Canton S ; tko25t
Cwol (BER1) ; tko25t
Recovery time (s)
50
40
30
20
10
0
a
b
b
a
a
b b
b b
a
♂
♀
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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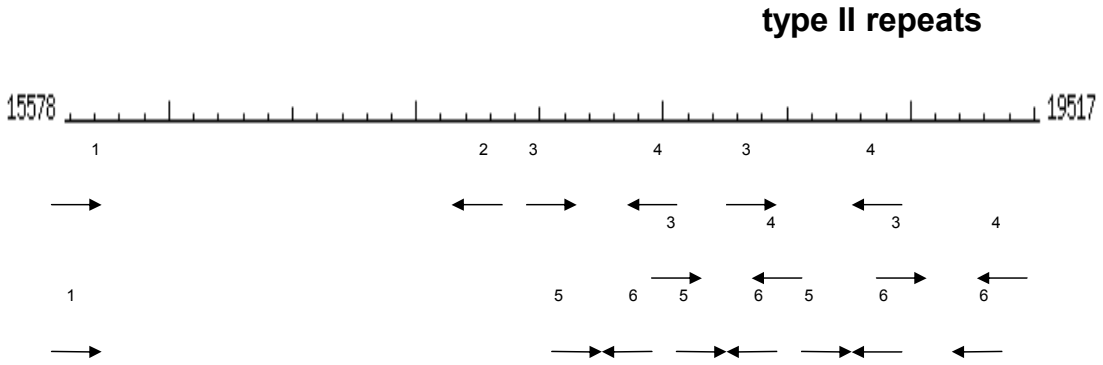
17556
SspI
PacI

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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       TTTAAAAATATTTTTTTTTTAAAA--AAAAATTTTTTTTTTAAAAAAAATTTTTTTTTTTAAAAAAATTTTAAAAAATTATAGATTAATTTCTTTTAAATG
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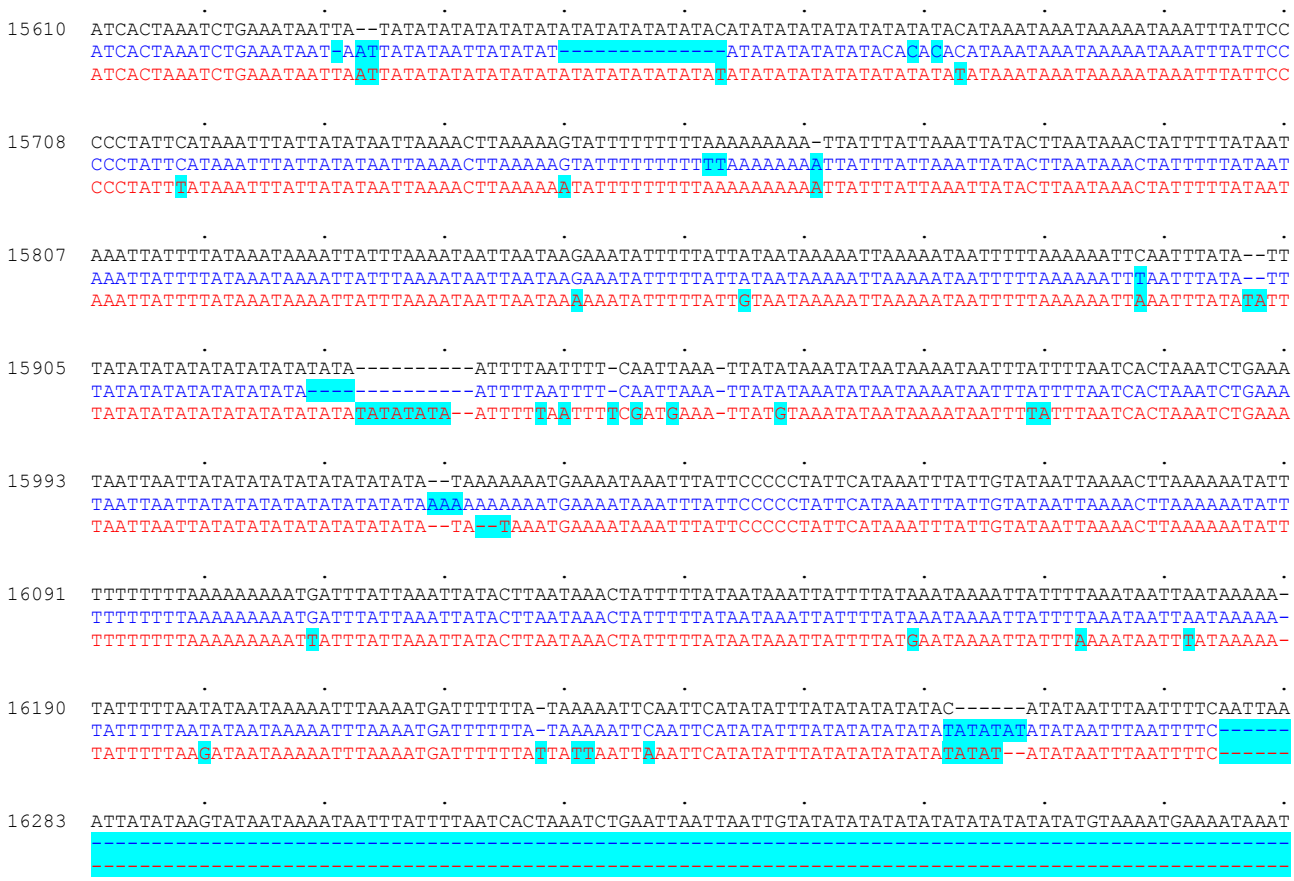

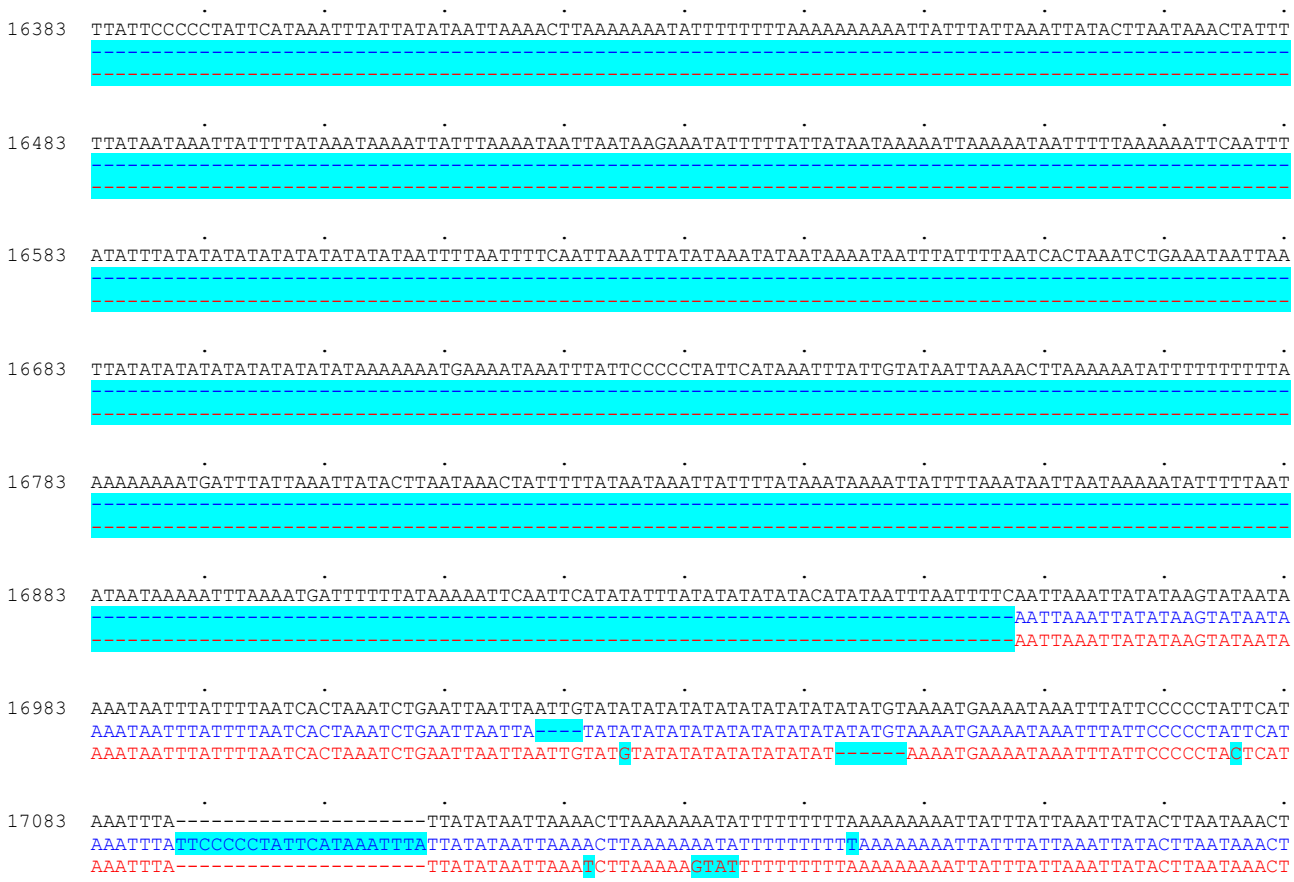
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16683 TTATATATATATATATATATATAAAAAAATGAAAATAAATTTATTCCCCCTATTCATAAATTTATTGTATAATTAAAACTTAAAAAATATTTTTTTTTTA
16783 AAAAAAAATGATTTATTAAATTATACTTAATAAACTATTTTTATAATAAATTATTTTATAAATAAAATTATTTTAAATAATTAATAAAAATATTTTTAAT
16883 ATAATAAAAATTTAAAATGATTTTTTATAAAAATTCAATTCATATATTTATATATATATACATATAATTTAATTTTCAATTAAATTATATAAGTATAATA
AATTAAATTATATAAGTATAATA
AATTAAATTATATAAGTATAATA
16983 AAATAATTTATTTTAATCACTAAATCTGAATTAATTAATTGTATATATATATATATATATATATATGTAAAATGAAAATAAATTTATTCCCCCTATTCAT
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17083 AAATTTA----------TTATATAATTAAAACTTAAAAAAATATTTTTTTTTAAAAAAAAATTATTTATTAAATTATACTTAATAAACT
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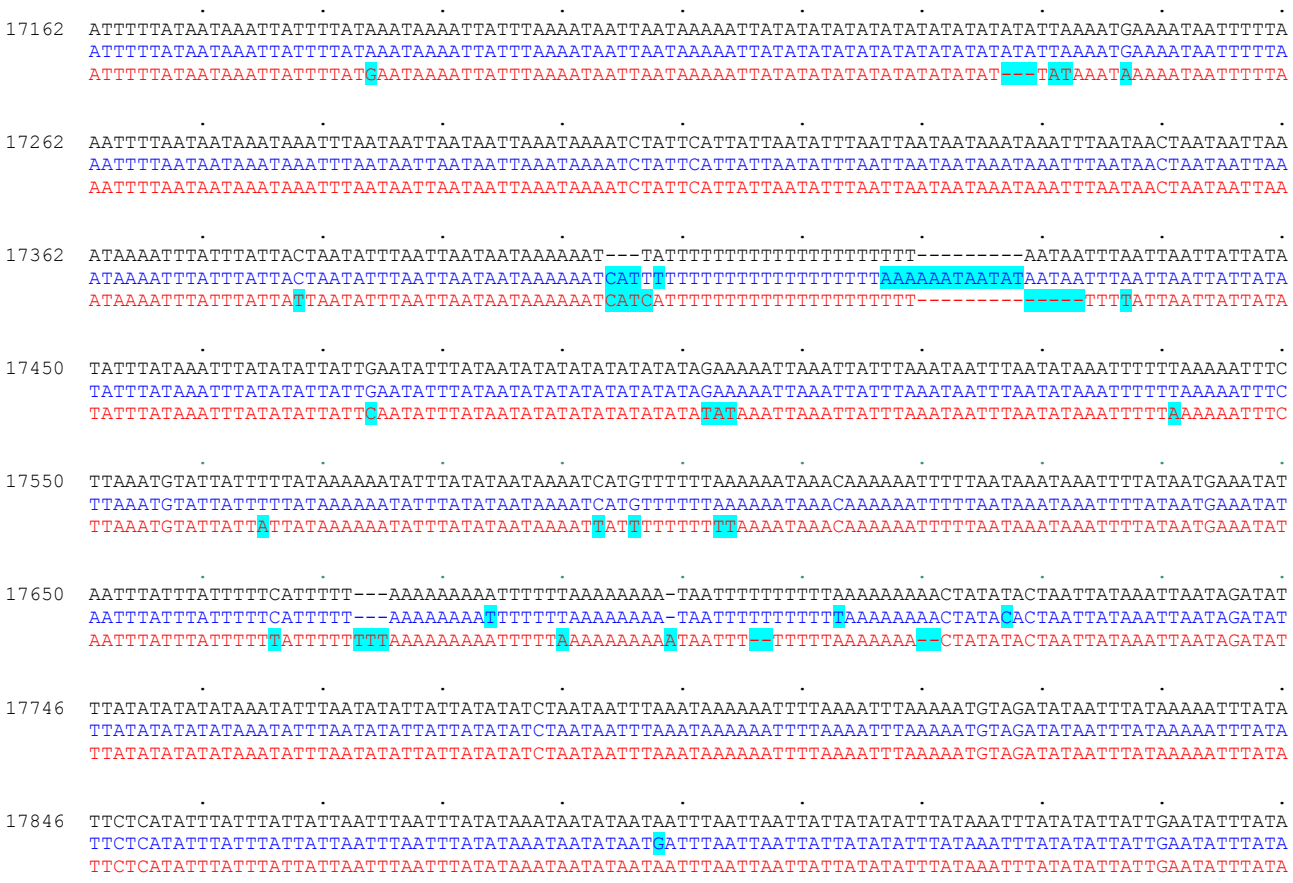

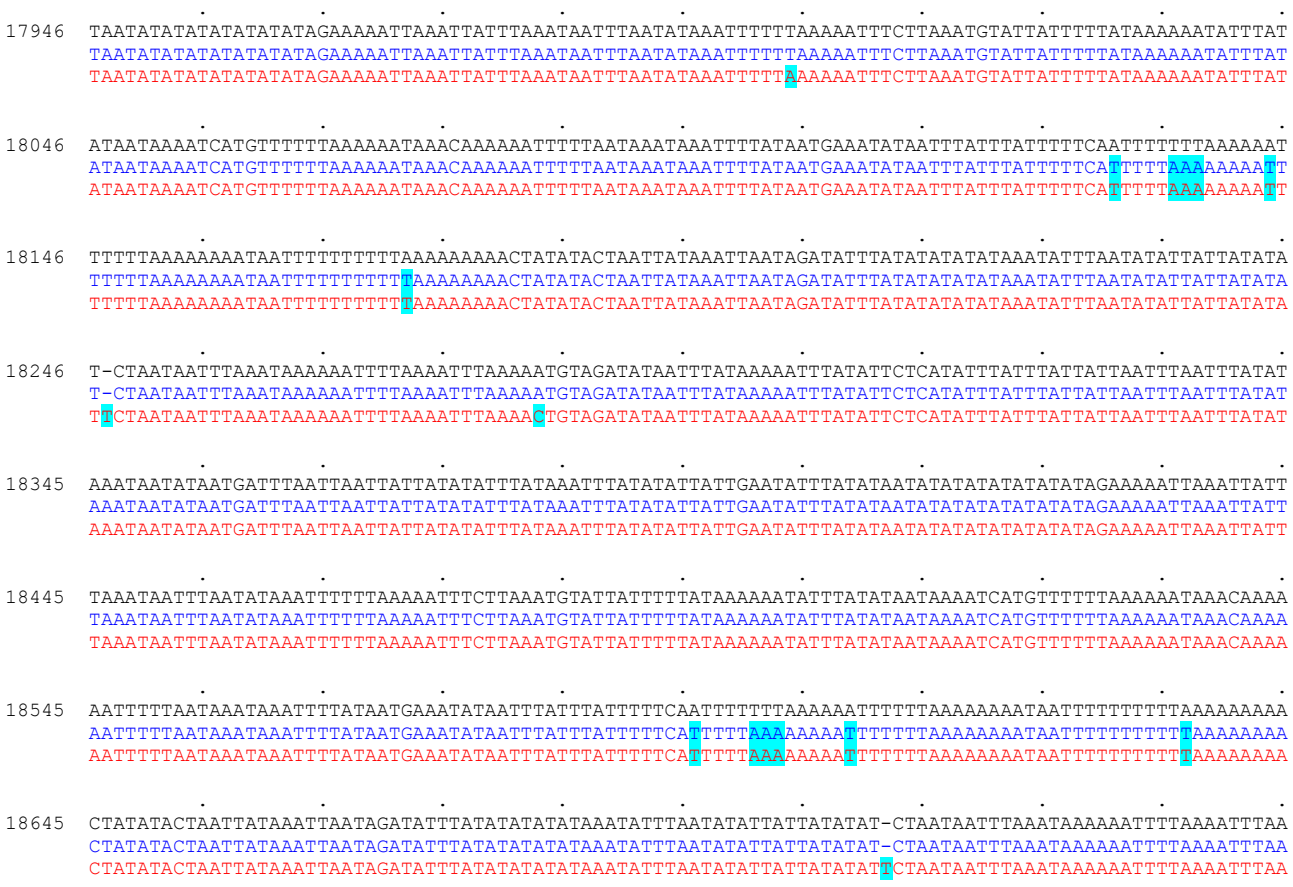
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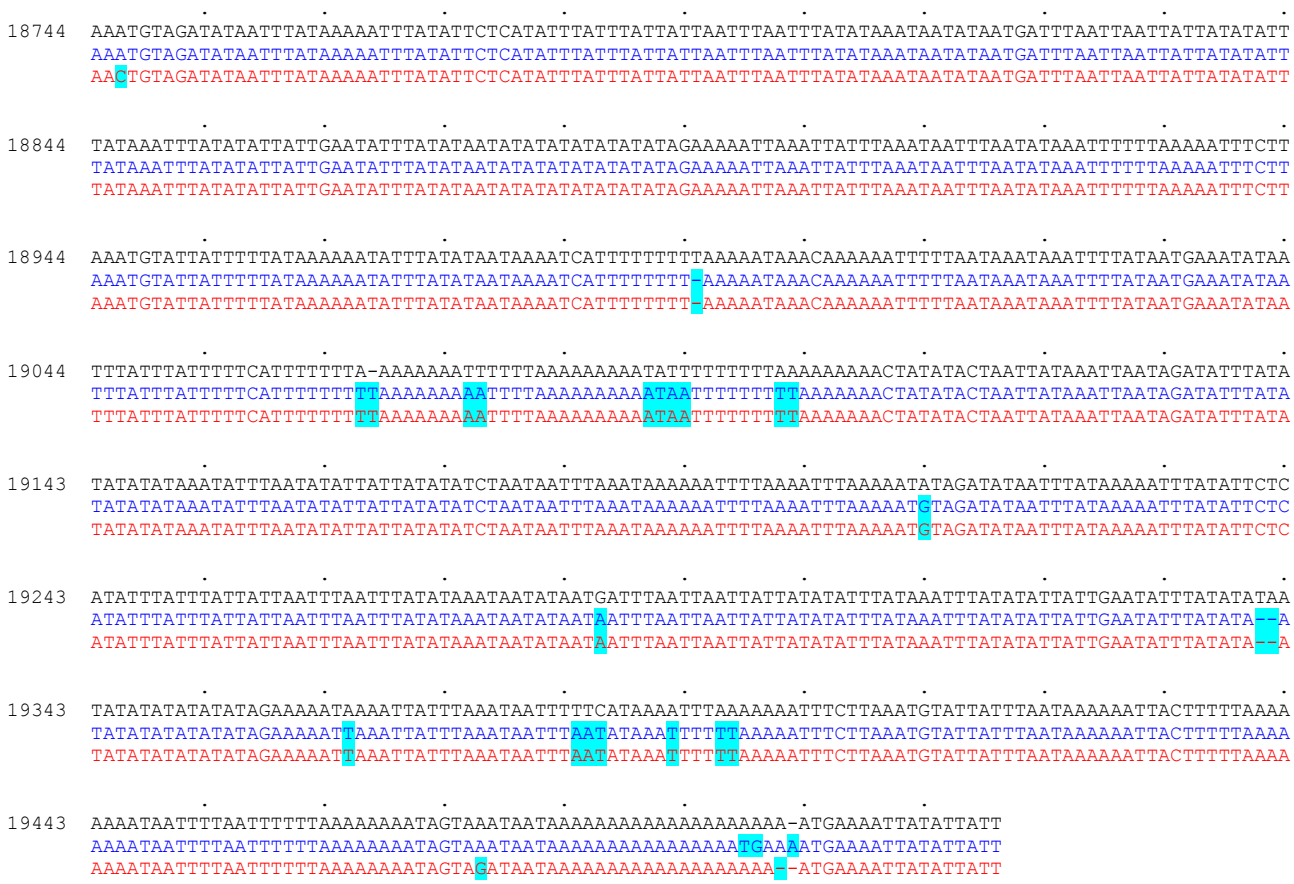
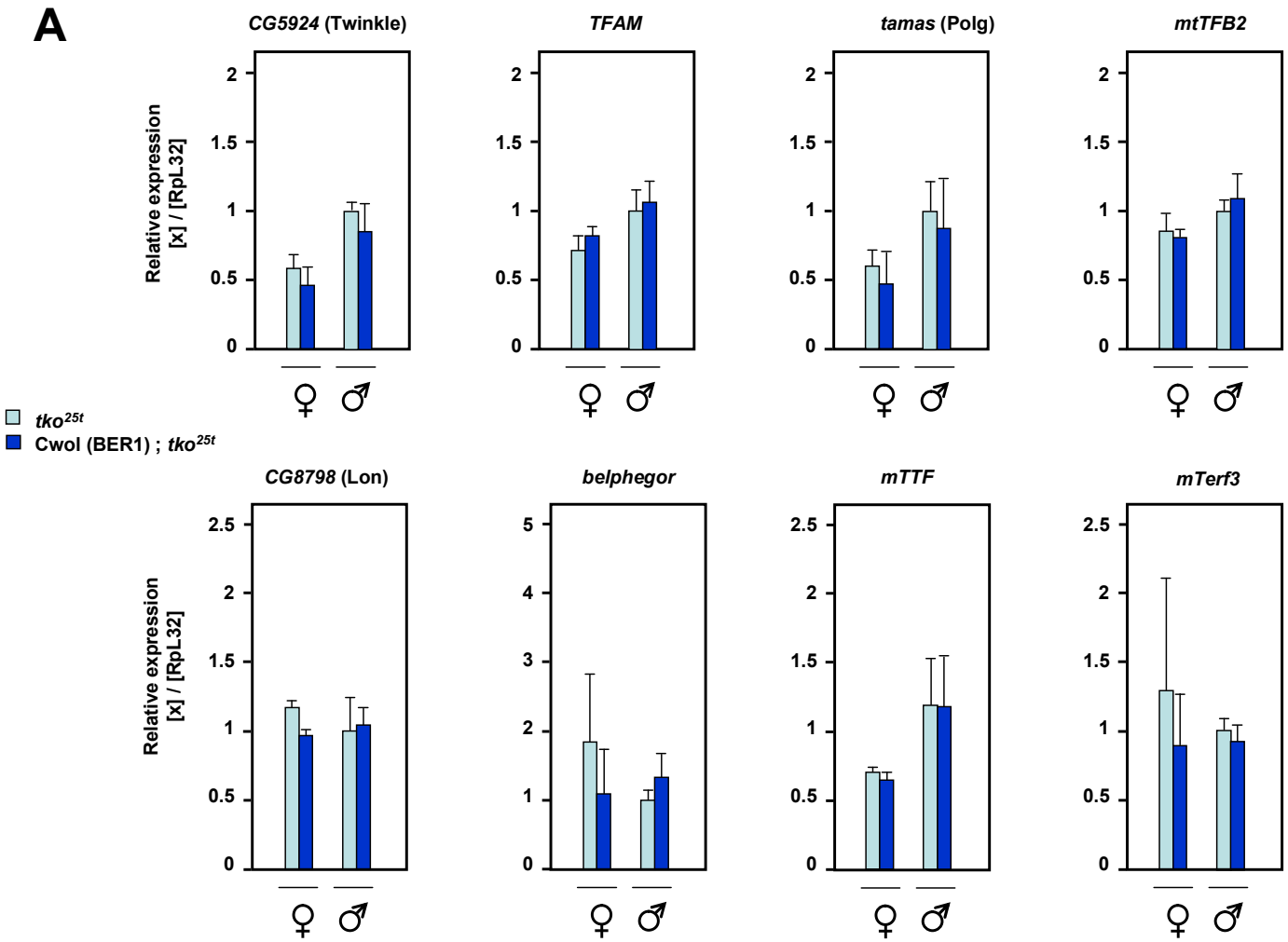


Figure S4 NCR structure and phenotypes conferred by mtDNAs of different *D. melanogaster* strains. (A) Phenotypic characterization of *tko*^{25t} flies in the Canton S and Oregon R mtDNA backgrounds, as indicated showing both to be fully of the non-suppressor type. a, b and c denote significantly different 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
♀
♂

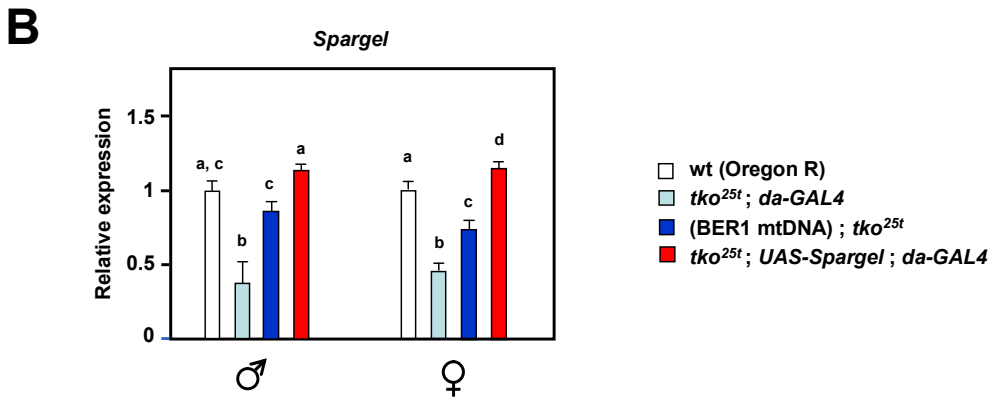


Figure S5 Expression levels of genes with known or proposed roles in mtDNA metabolism in *tko*^{25t} flies, in different mtDNA backgrounds. Q-RT-PCR of the indicated mRNAs, normalized to that of *RpL32* and then to the values for *tko*^{25t} females in the original (Oregon R-related) strain background, 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′

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.143719/-/DC1

A Cytoplasmic Suppressor of a Nuclear Mutation Affecting Mitochondrial Functions in *Drosophila*

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DOI: 10.1534/genetics.112.143719

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

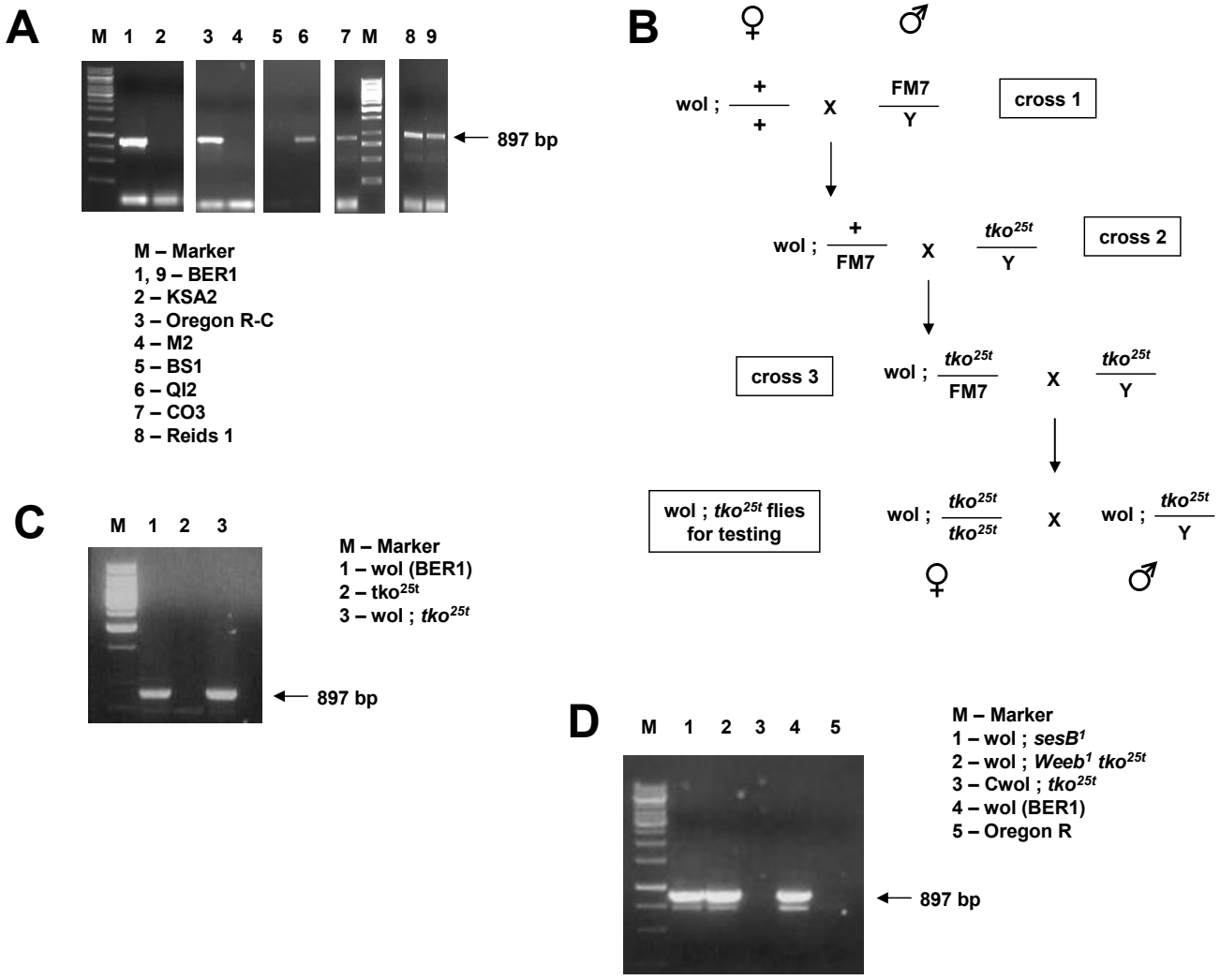
enzyme and 3 µl (30 µCi) of labeled ATP. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1 µl of 0.5 M EDTA (pH 8.0) and further incubation at 75 °C for 10 min. The unincorporated label was removed by gel filtration on Sephadex G-50 (GE healthcare). The mtDNA noncoding 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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M 1 2 3 4 5 6 7 M 8 9
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

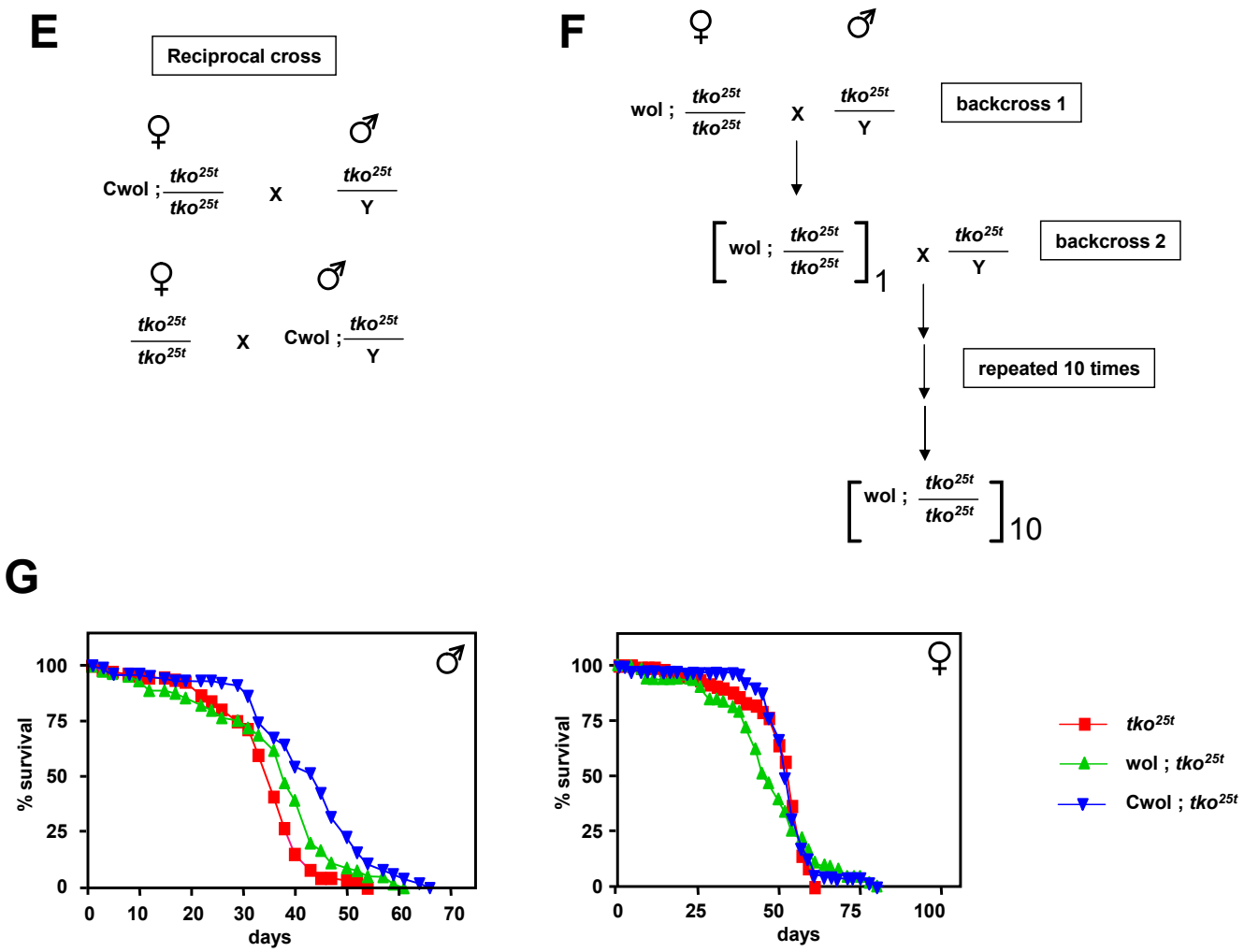
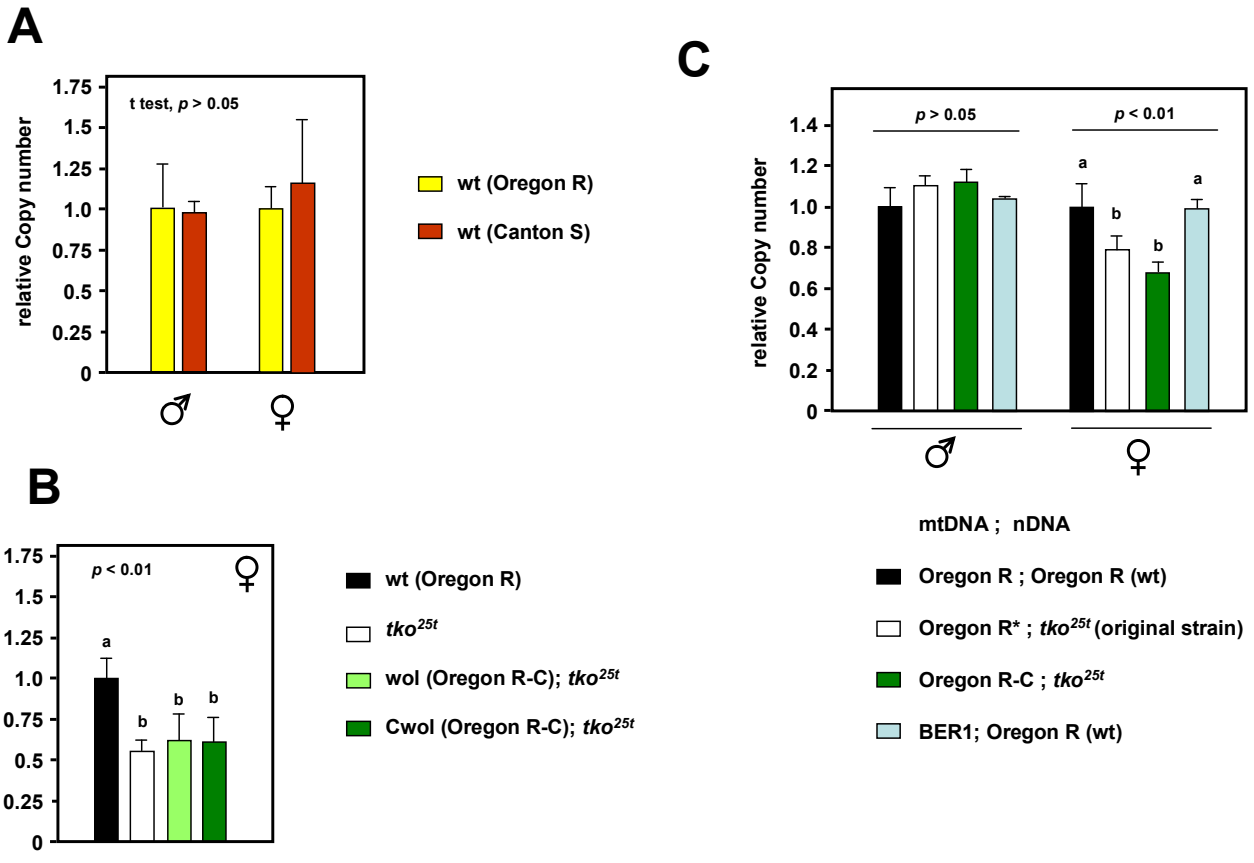


Figure S1 Creation and phenotypic characterization of cybrid *tko*^{25t} and *sesB*^{1} flies. (A) Representative gels of PCR products obtained using the *Wolbachia* 16S rRNA gene primers on genomic DNA of single individuals from the wild-type strains indicated. Gels run in parallel using the same marker 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
♂
♀
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Oregon R ; Oregon R (wt)
Oregon R* ; tko25t (original strain)
Oregon R-C ; tko25t
BER1; Oregon R (wt)

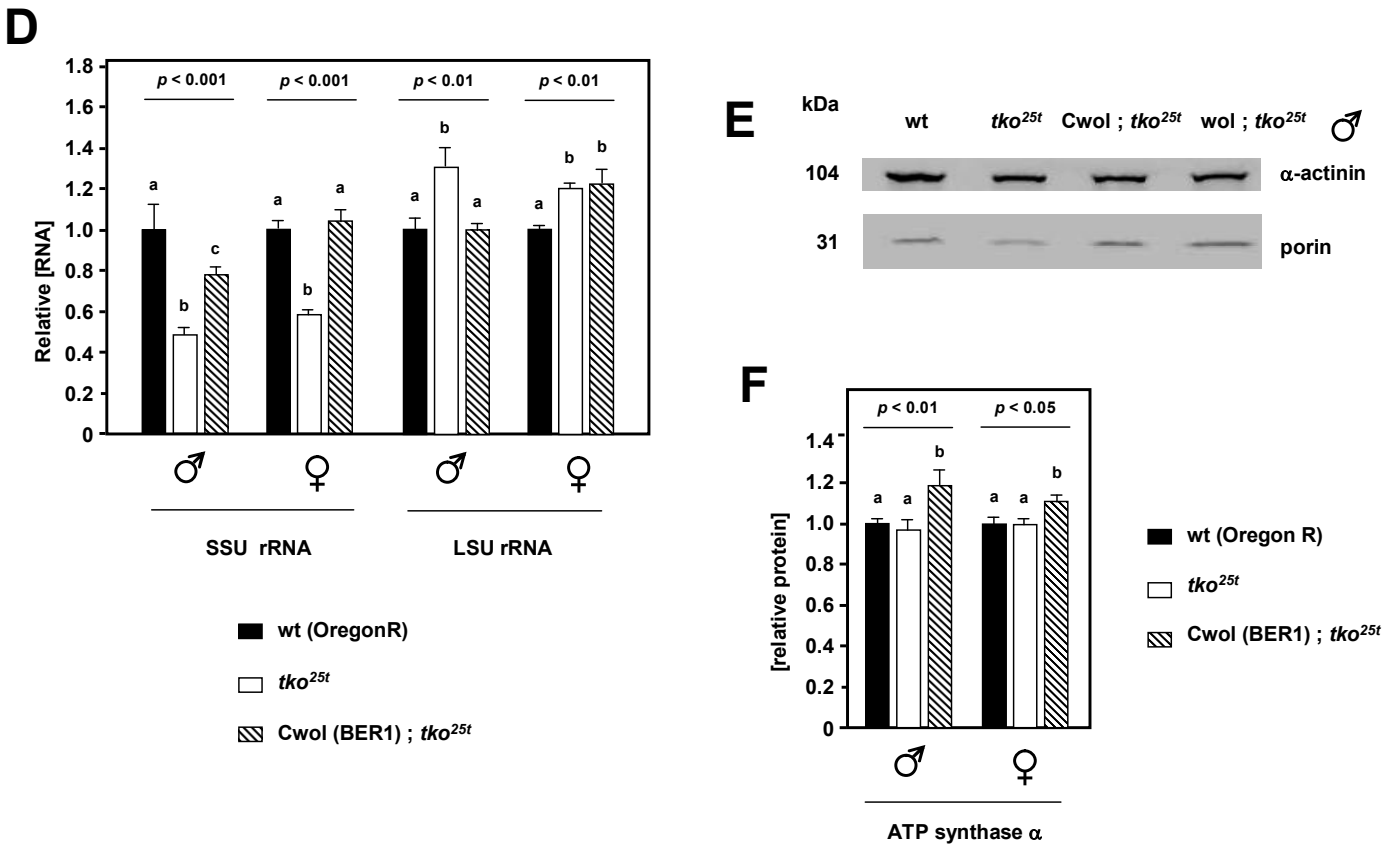


Figure S2 Molecular correlates of partial suppression of *tko*^{25t} by the suppressor mtDNA backgrounds. (A) Supplementary data to Fig. 2D: copy number of mtDNA in two different wild-type strains is similar. Copy number, relative to 18S rDNA, means ± SD, was normalized to that in Oregon R flies of the 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).

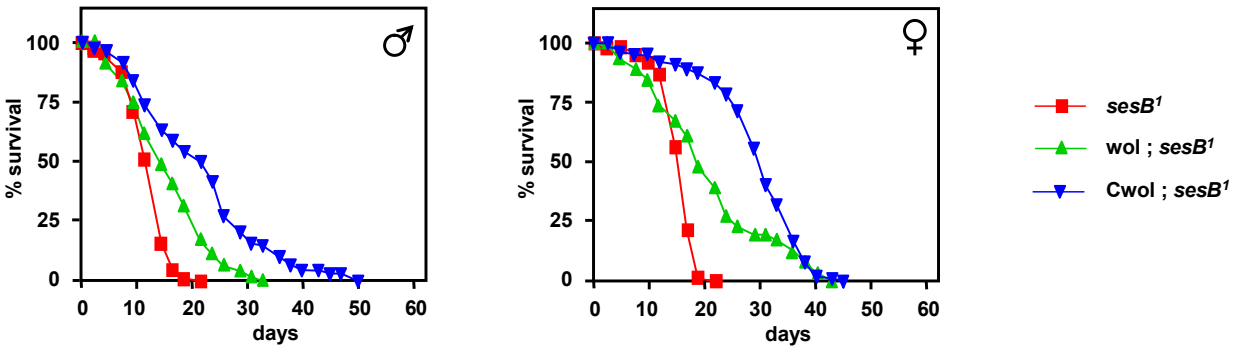
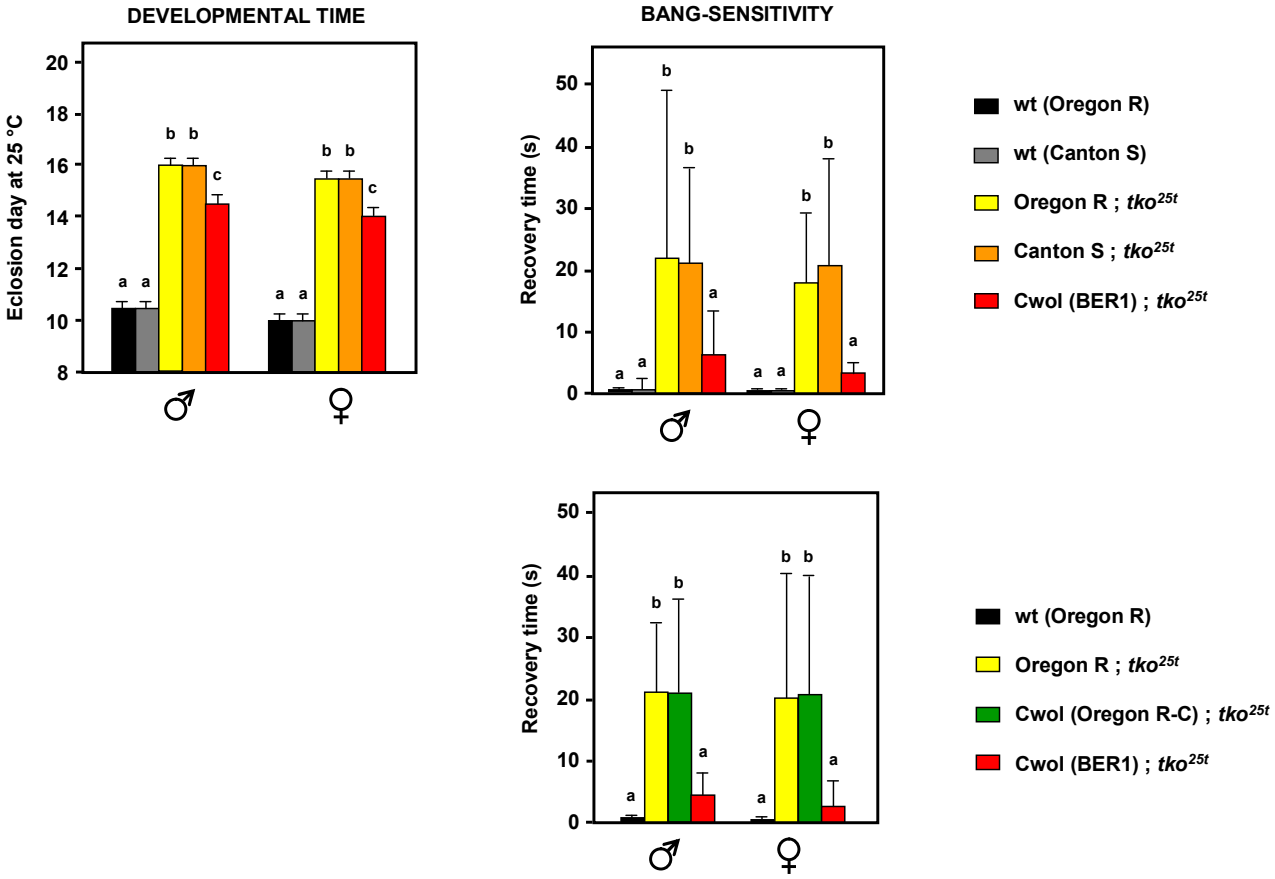
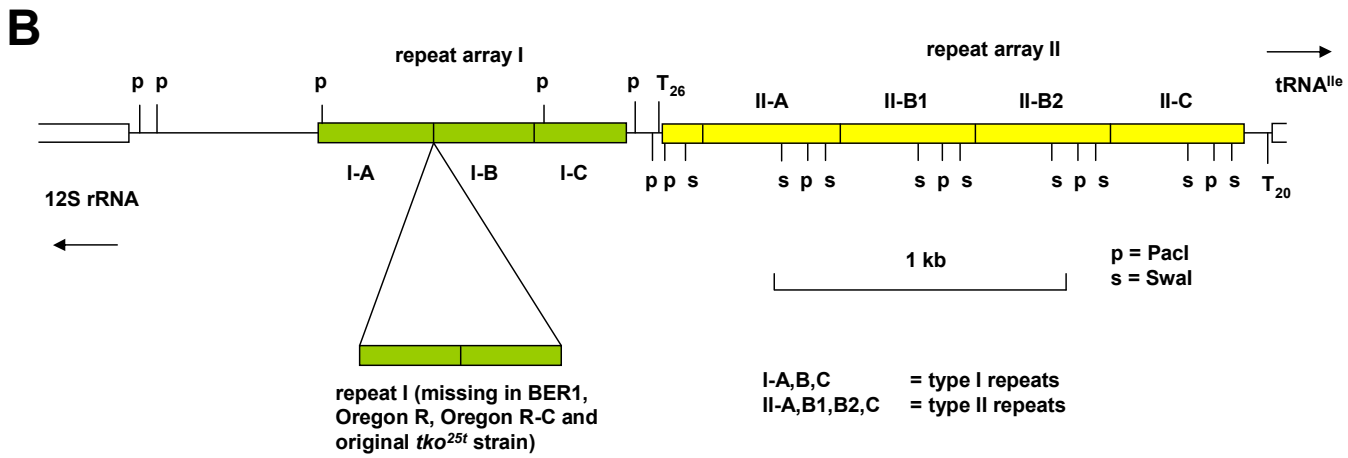
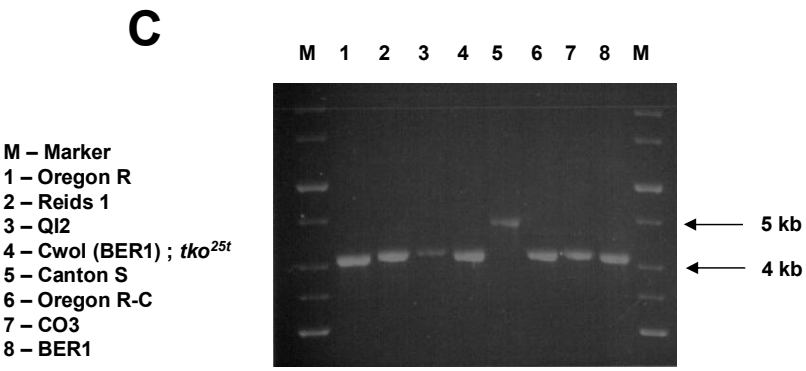


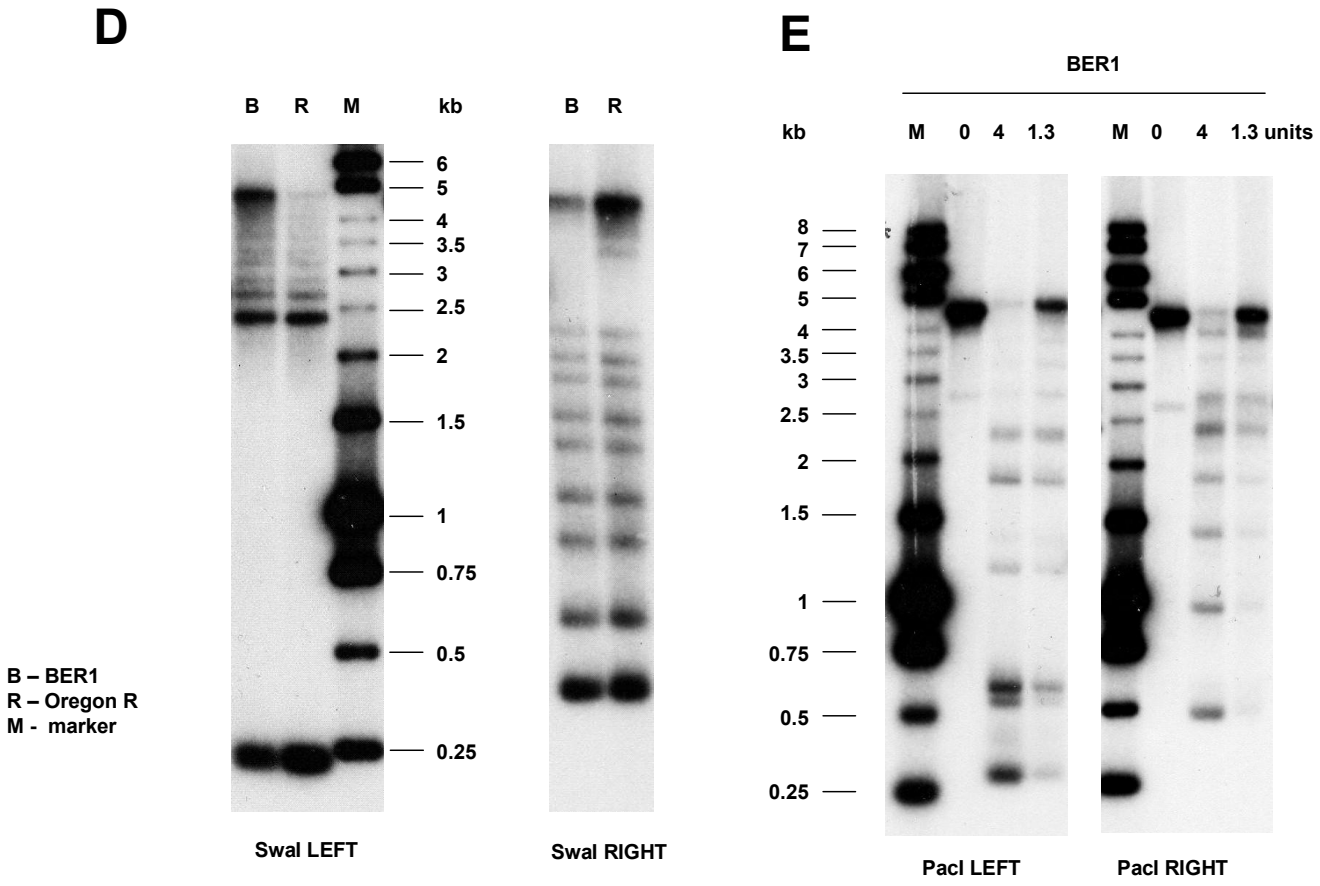
Figure S3 BER1 strain mtDNA increases the lifespan of *sesB*^{1} flies. Lifespan curves for flies of the genotypes indicated. For both sexes, the curves for wol (BER1); *sesB*^{1} and Cwol (BER1) ; *sesB*^{1} (median survival of 17, 19 d, respectively, for males, 31 and 26 d for females) were significantly 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

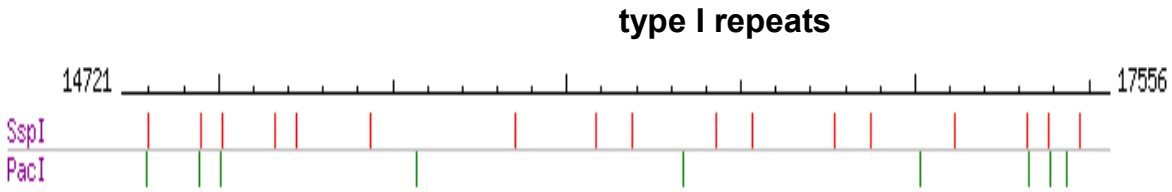

DEVELOPMENTAL TIME
Eclosion day at 25 °C
20
18
16
14
12
10
8
a a
b b
c
a a
b b
c
♂
♀
BANG-SENSITIVITY
Recovery time (s)
50
40
30
20
10
0
a a
b
b
a
a a
b
b
a
♂
♀
wt (Oregon R)
wt (Canton S)
Oregon R ; tko25t
Canton S ; tko25t
Cwol (BER1) ; tko25t
Recovery time (s)
50
40
30
20
10
0
a
b
b
a
a
b b
b b
a
♂
♀
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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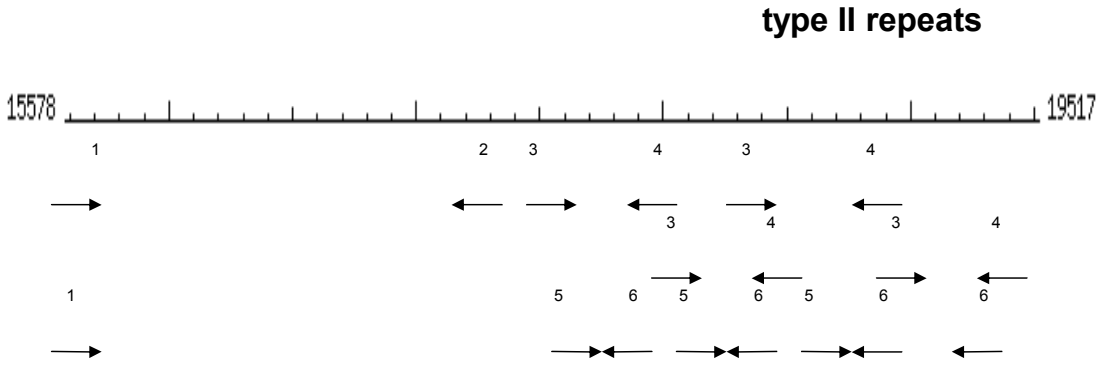
17556
SspI
PacI

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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       ATTTTTATTTTTGATTTTTTATTATTAATTAAATATTAATAATTTTTATAAAAATAATTTTTTTAAGAAAAATTAAAATTAATTTTAATTAAATATTAAA
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       TTTAAAAATATTTTTTTTTTAAAA--AAAAATTTTTTTTTTAAAAAAAATTTTTTTTTTTAAAAAAATTTTAAAAAATTATAGATTAATTTCTTTTAAATG
       TTTAAAAATATTTTTTTTTTAAAAAAAAAAAATTTTTTTTTTAAAAAAATTTTTTTTTTTAAAAAAATTTTAAAAAATTATAGATTAATTTCTTTTAAATG

                .         .         .         .         .         .         .         .         .         .
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       ACTAAAAAAAATTTTTTTTTTTAAGTATTTTAAAACTTTTTTTTTACAATTTTTTAAAAAATATATAAATATAAATTTTAAAAATT--TTTTTTTTTTAA
       ACTAAAAAAAAAATTTTTTTTTTAAGTATTTTAAAACTTTTTTTTTACAATTTTTAAAAAAATATATAAATATAAATTTTAAAAATTTTTTTTTTTTAAA

                .         .         .         .         .         .         .         .         .         .
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                .         .         .         .         .         .         .         .         .         .
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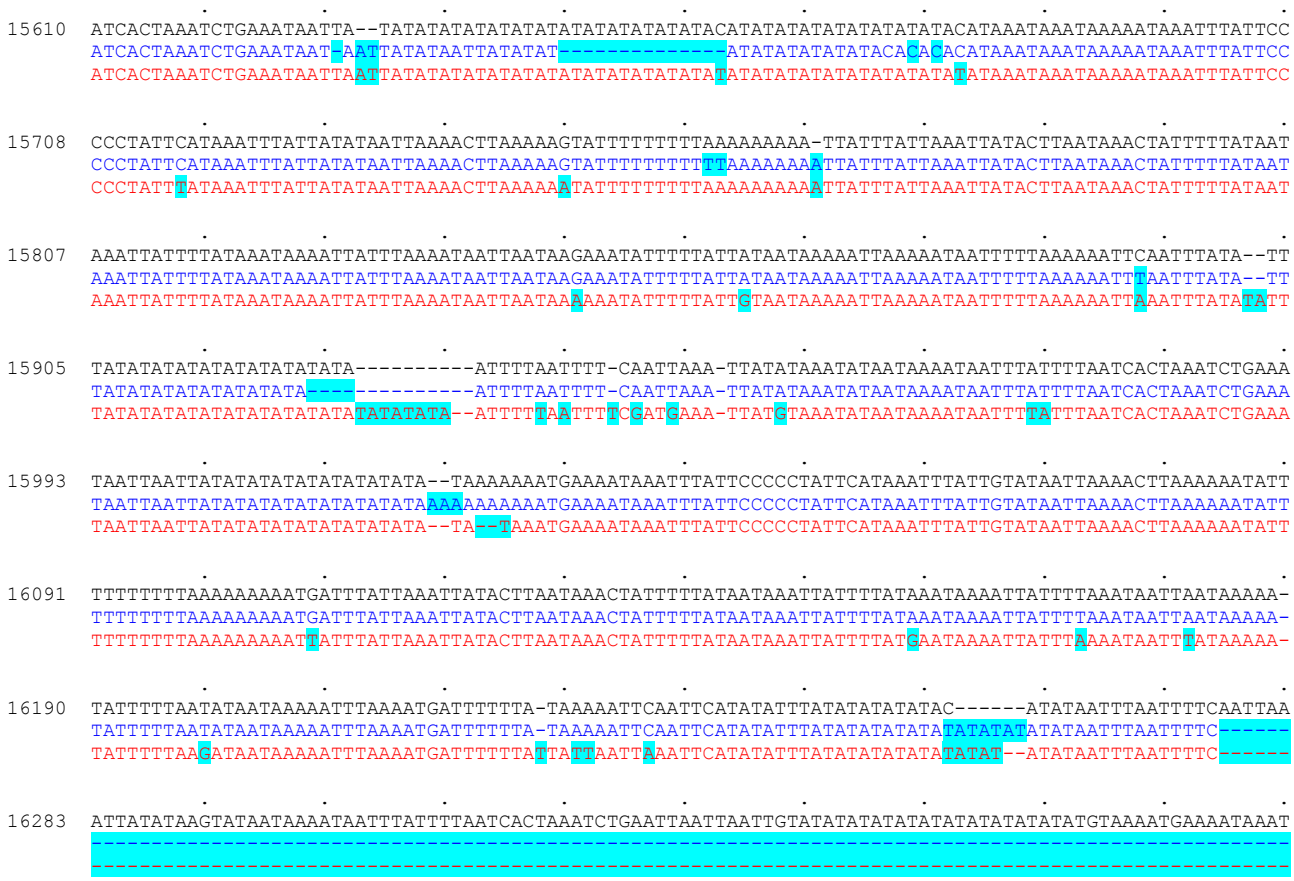

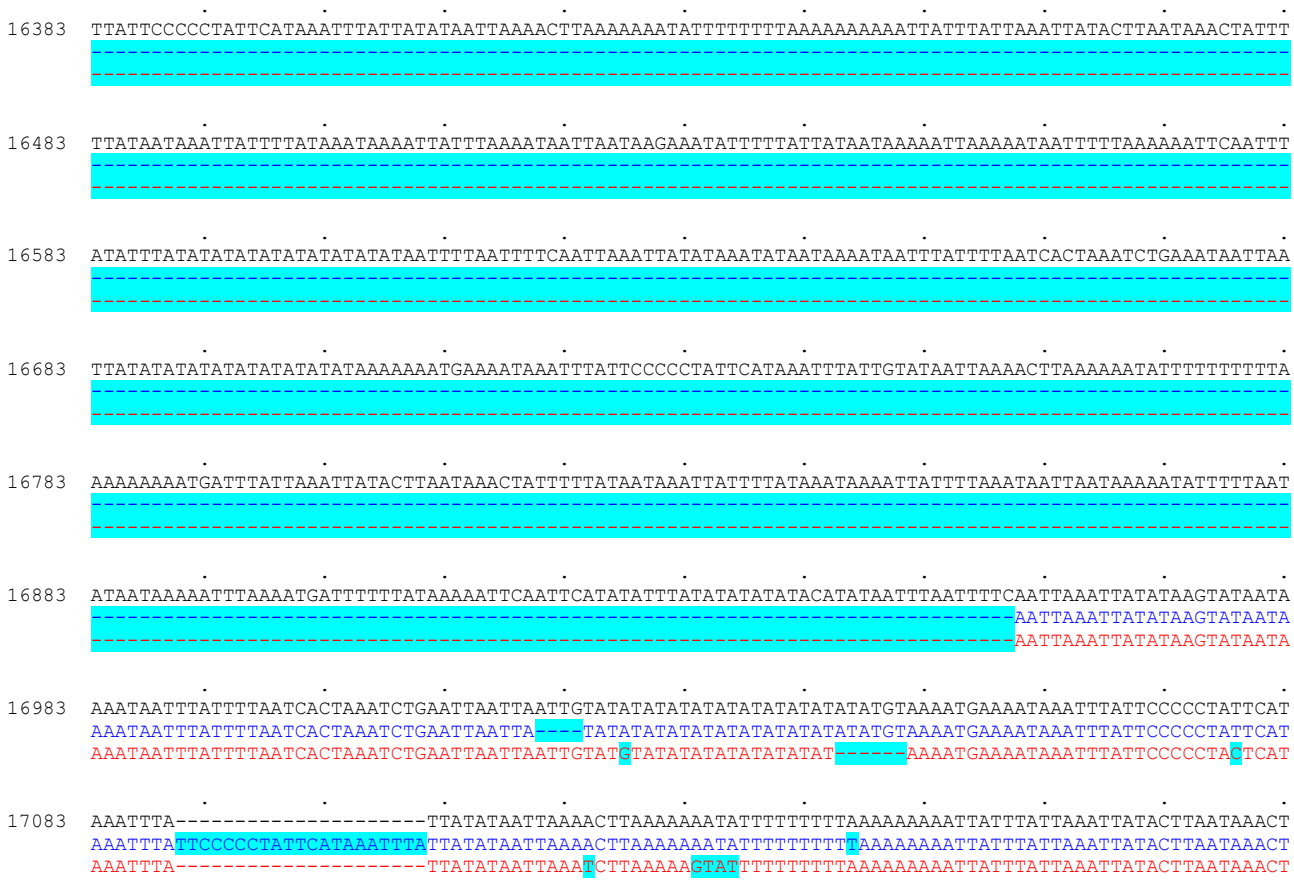
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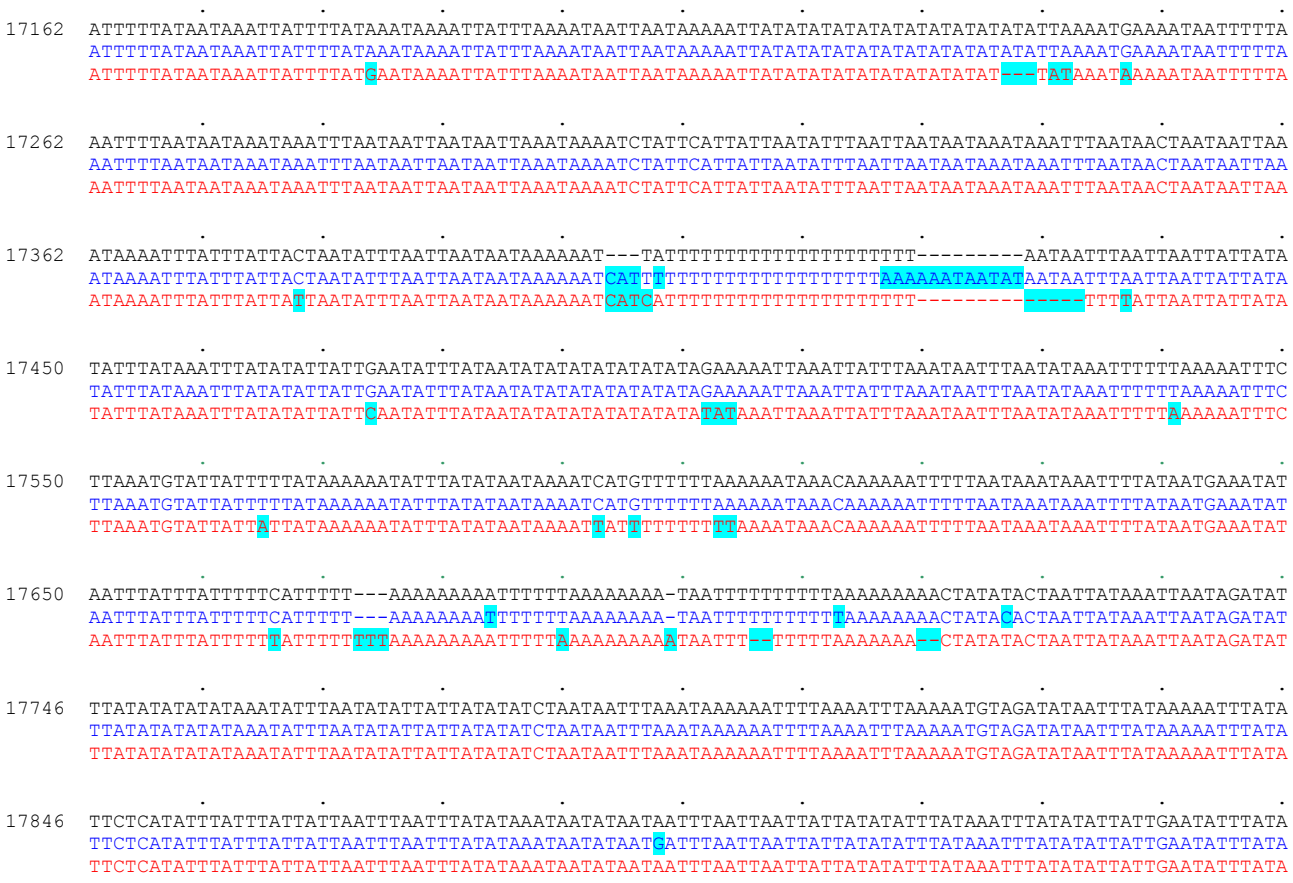

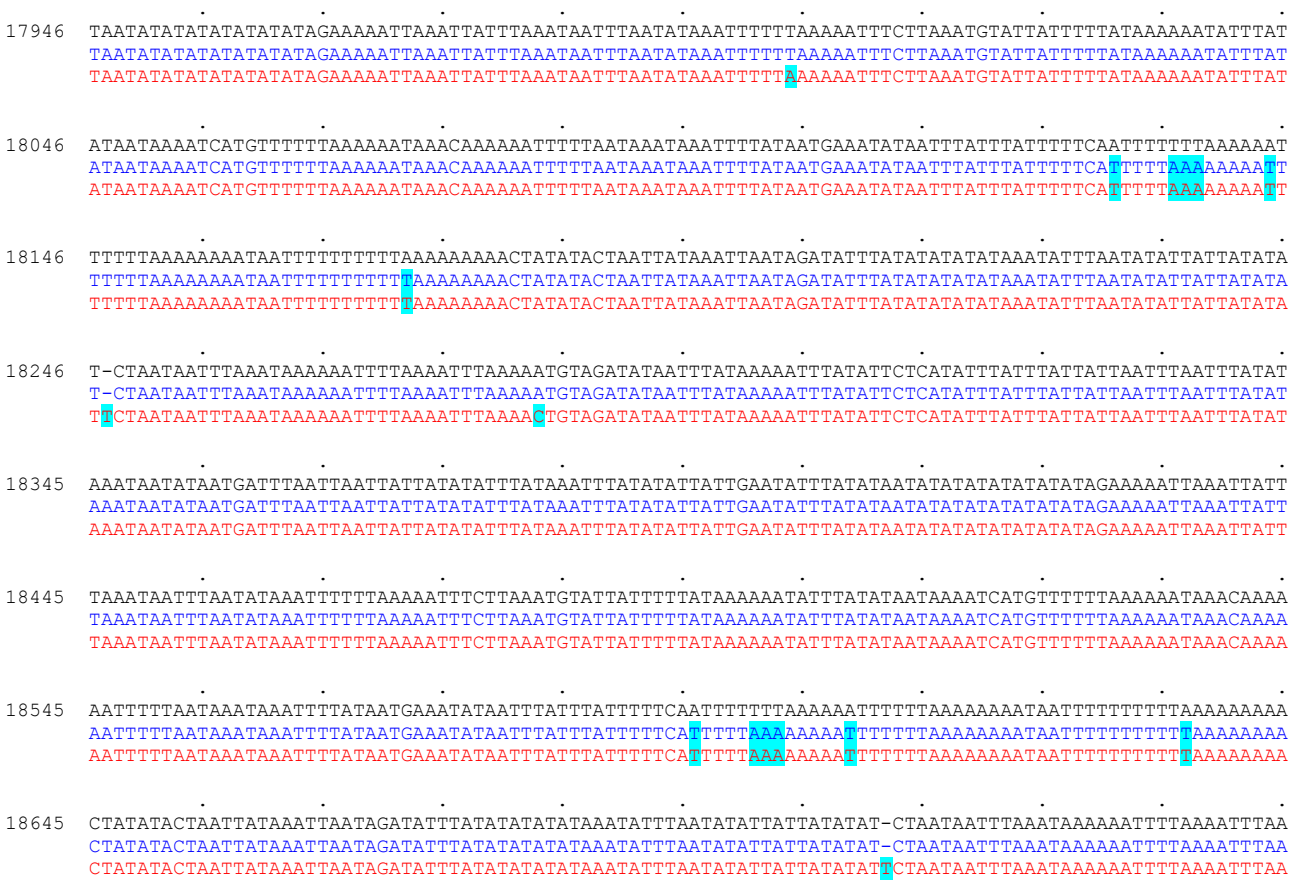
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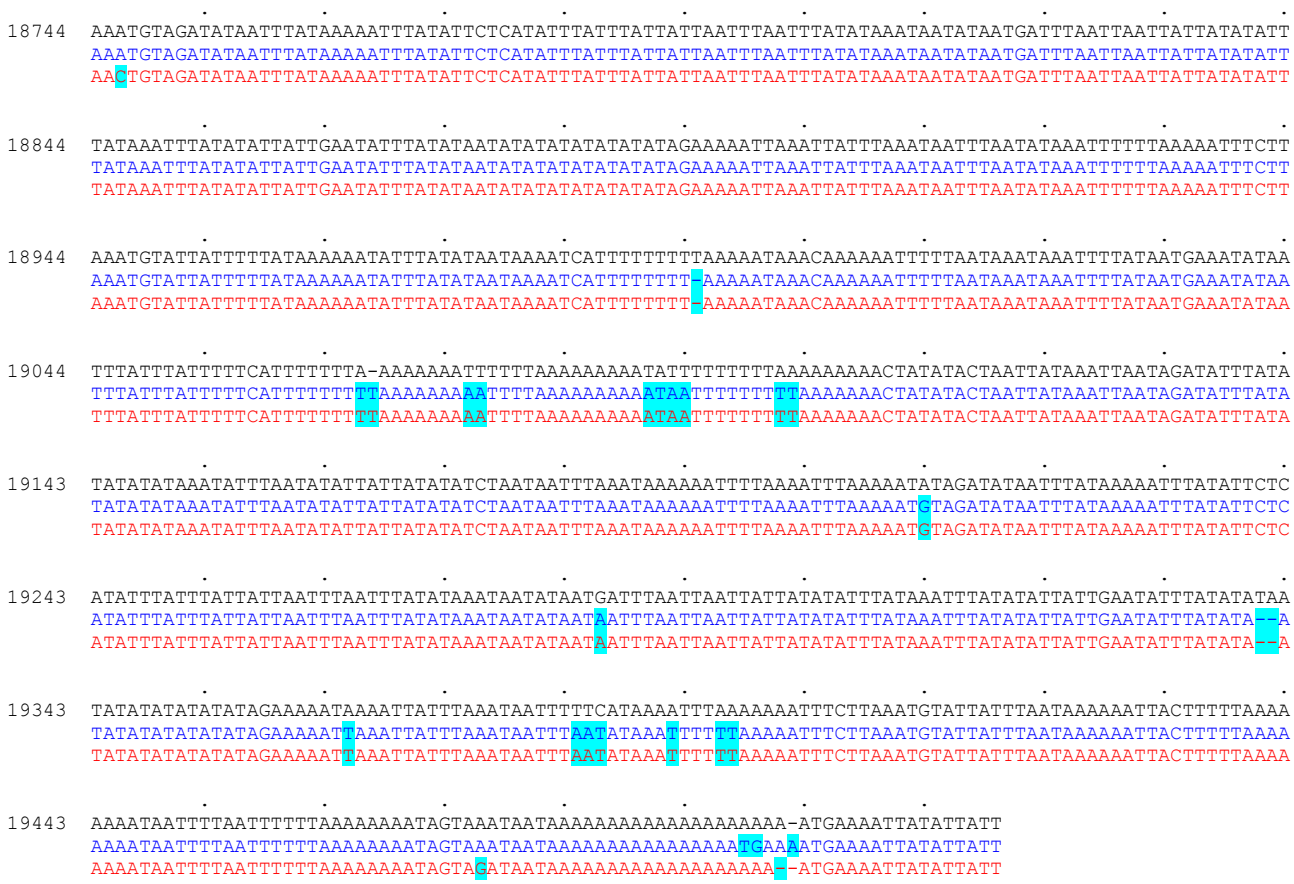
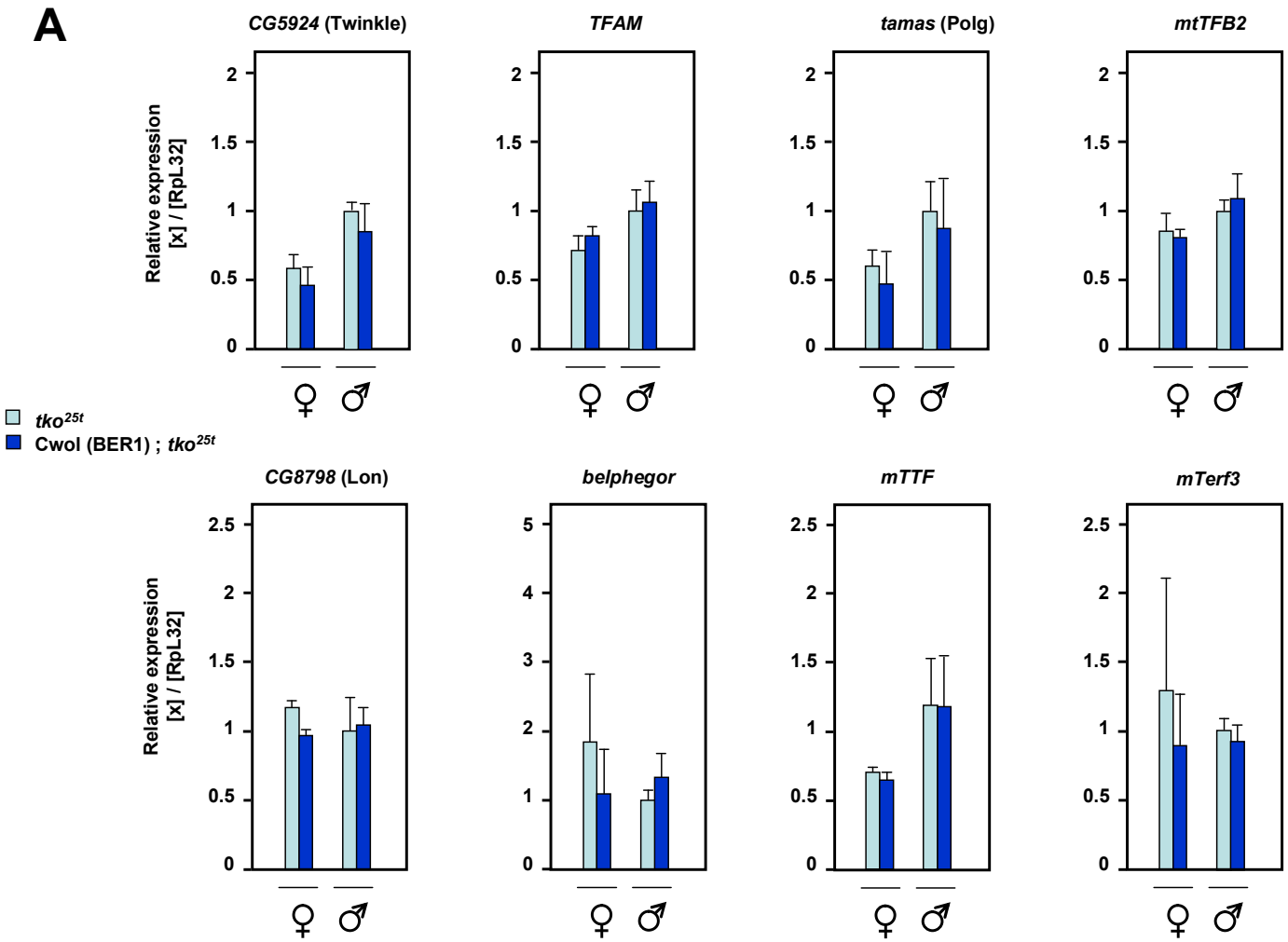


Figure S4 NCR structure and phenotypes conferred by mtDNAs of different *D. melanogaster* strains. (A) Phenotypic characterization of *tko*^{25t} flies in the Canton S and Oregon R mtDNA backgrounds, as indicated showing both to be fully of the non-suppressor type. a, b and c denote significantly different 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
♀
♂

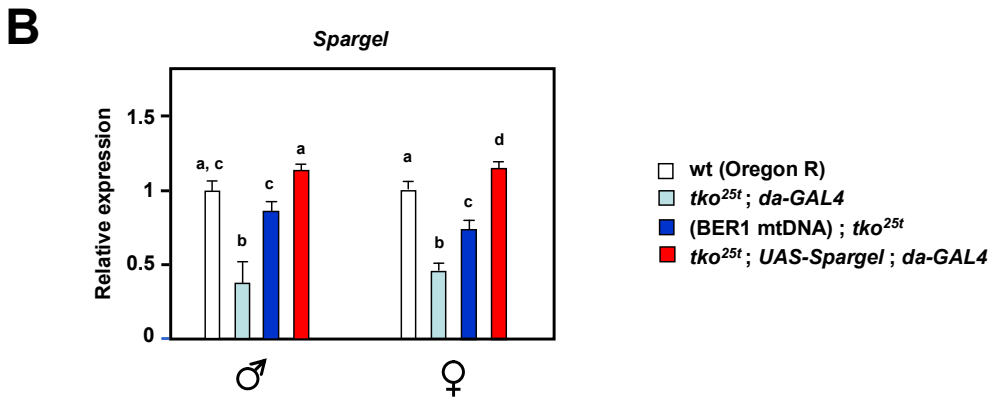


Figure S5 Expression levels of genes with known or proposed roles in mtDNA metabolism in *tko*^{25t} flies, in different mtDNA backgrounds. Q-RT-PCR of the indicated mRNAs, normalized to that of *RpL32* and then to the values for *tko*^{25t} females in the original (Oregon R-related) strain background, 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′