SUPPLEMENTARY MATERIAL

MATERIAL AND METHODS

In vitro priming on ssDNA oligonucleotides

The reaction mixture contained 100 fmol ssDNA oligonucleotide, 10 mM Tris-HCl [pH 8.0], 25 mM MgCl2, 1 mM DTT, 100 μg/ml BSA, 400 μM ATP, 150 μM CTP, 10 μM GTP, 150 μM UTP, (α-32P) 1mM GTP, 4 units RNase inhibitor (Amersham Biosciences), 500 fmol of POLRMT and 1 pmol of mtSSB. After 30 min incubation at 32 ºC, 12 units of RNase free DNase I (Qiagen) was added. Reactions were processed as previously described for *in vitro* transcription reactions [1] and analyzed on a 25 % polyacrylamide gel containing 3 M urea in 1 × TBE.

Rolling-circle mtDNA replication

Reactions were carried out as described previously [2] unless indicated otherwise, in the presence of [α-32P] dCTP in order to label newly synthesized DNA. For rolling-circle template formation, we cloned a DNA fragment corresponding to nts 5275–6203 of the mitochondrial human genome between the HindIII and EcoRI sites in the pBluescript SK(+) vector (Agilent Technologies; La Jolla, CA). The pBluescript SK(+)OriL construct was used as a template in site-directed PCR mutagenesis reactions to generate the mutant variants of OriL. The mouse OriL and mutant variants were obtained by PCR amplification of a region in the mouse mitochondrial genome corresponding to nts 4921-5521. As template we used used the clones obtained for sequence analysis of mouse mtDNA genomes. The fragments obtained were cloned between the HindIII and EcoRI sites in pBluescript SK(+). Constructs were confirmed by sequencing and used for ssDNA isolation following the manufacturer's protocol (Stratagene). To produce the rolling-

circle DNA replication template, we annealed a 70-mer oligonucleotide (20 pmol) (5′-42[T]-ATC TCA GCG ATC TGT CTA TTT CGT TCA T-3′) to the pBluescript SK(+)OriL ssDNA (2 pmol) and the second strand was synthesized in a PCR reaction using KOD Hot Start DNA polymerase (Novagen). The samples were purified using the QIAquick PCR Purification Kit (QIAGEN). The reaction mixtures (20 μL) contained 10 fmol of the dsDNA template (wt or mutant versions) in the presence of 150 μM UTP, 150 μM GTP, 150 μM CTP, TWINKLE (100 fmol), POLγA (250 fmol), POLγB (375 fmol, calculated as dimer), mtSSB (5 pmol), and POLRMT (250 fmol) if nothing else is indicated in the figure legends. We stopped the reactions after 60 min (or at the times indicated in the figures) at 37 ºC by adding 6 μL of stop alkaline gel loading buffer and the samples were analyzed as described before [1, 3, 4].

Southern Blot analysis:

Rolling circle reactions and Southern blot analysis was carried out as described previously [2] using radioactively labeled 50-mer probes. Primer sequences are available upon request.

Mutation Load Analysis:

For sequencing of mtDNA, we isolated tissue from 8 genetically modified mice, carrying a proofreading deficient form of the mitochondrial DNA polymerase γ (PolγA^{D257A}/PolAγA^{D257A}). that display increased overall levels of somatic mtDNA point mutations [5]. Total DNA was extracted from liver, large intestine or from tail biopsy samples obtained from the mice at weaning (3 weeks of age). The target region for the analysis spanned the 3’ end of the mt-nad2 gene to approximately the midpoint of the mt-co1 gene (primers N2-H4921 5′-CCT ACC CCT

AGC CCC CC-3′ and C1-L5953 5′-AGT ATA GTA ATG CCT GCG GC-3′). DNA was amplified using the Phusion® High-Fidelity DNA Polymerase (Finnzymes).

PCR products were cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). Plasmids were purified by the NucleoSpin® 96 Plasmid (Machery-Nagel) on a vacuum-equipped Microlab® Starlet system (Hamilton Robotics). Sequencing was conducted using vector-encoded M13F and M13R primers, using Big Dye v3.1 chemistry. Sequencing products were purified using the BigDye® Xterminator™ Purification Kit, and resolved on a 3730 DNA Analyzer (Life Technologies – Applied Biosystems). 974 bp of consistently clean clone sequence was used in this analysis (positions 4950-5923 on the reference sequence from GenBank Accession JF286601.1). A total of 1747 clones (≈1,700,000 bp of sequence) were used in this study. For analysis of the polyT region within OriL (11 dA's at positions 5172-5182 on the reference genome), 346 clones were determined to give reads of sufficient clarity for two-strand confirmation of the number of encoded dT's. To test if the observed decrease in mutation load at OriL was statistically significant, we generated 1000 data sets, each by randomly selecting 32 non-OriL sites from our sequencing data. We then compared the mean per-site mutation rate of each simulated data set to that observed for OriL. The observed per-site mutation load in OriL was lower than in 968 of the 1000 random data sets analyzed ($\alpha = 0.05$, 2 tailed t-test), demonstrating that the OriL sequence differed significantly from the surrounding sequences. To determine the background error rate for the assay, a single clone containing 3 SNPs was chosen, diluted into *Drosophila melanogaster* total DNA extract, and amplified, cloned and sequenced as above. Of 295 clones assayed, only one variation from the reference sequence was observed (error rate of 3.48×10^{-6}) and no variation in the number of dA's were observed.

REFERENCES

1. Gaspari M, Falkenberg M, Larsson NG, Gustafsson CM (2004) The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells. *The EMBO journal* **23:** 4606-4614

2. Fuste JM *et al* (2010) Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication. *Molecular cell* **37:** 67-78

3. Korhonen JA, Pham XH, Pellegrini M, Falkenberg M (2004) Reconstitution of a minimal mtDNA replisome in vitro. *The EMBO journal* **23:** 2423-2429

4. Wanrooij S, Fuste JM, Farge G, Shi Y, Gustafsson CM, Falkenberg M (2008) Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 11122-11127

5. Trifunovic A *et al* (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429:** 417-423

Supplementary table S1. OriL and mutant OriL derivatives used in this study.

Name	Modification	Sequence 5' to 3'
Wt	Wild-Type	GGGCTTCTCCCGCCTTTTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
Stem +6	Duplication 5735-40 and 5753-58	GGGCTTCTCCCGCCCCCGCCTTTTTTCCCGGCGGCGGGGGCGGGAGAAGTAGATTG
Stem +3	Duplication 5738-40 and 5753-56	GGGCTTCTCCCGCCGCCTTTTTTCCCGGCGGCGGCGGGAGAAGTAGATTG
Stem -1	Deletion 5735 and 58	GGGCTTCTCCGCCTTTTTTCCCGGCGGCGGAGAAGTAGATTGAAGCC
Stem -2	Deletion 5735-36 and 5757-58	GGGCTTCTCGCCTTTTTTCCCGGCGGCGAGAAGTAGATTGAAGCCAG
Stem -3	Deletion 5735-37 and 5756-58	GGGCTTCTGCCTTTTTTCCCGGCGGCAGAAGTAGATTGAAGCCAGTT
Stem -4	Deletion 5734-37 and 5756-59	GGGCTTCGCCTTTTTTCCCGGCGGCGAAGTAGATTGAAGCCAGTT
Stem -5	Deletion 5732-36 and 5757-61	GGGCTCGCCTTTTTTCCCGGCGGCGAGTAGATTGAAGCCAGTT
Stem -6	Deletion 5732-36+5740 and 5757-61+5753	GGGCTCGCTTTTTTCCCGGCGCGAGTAGATTGAAGCCAGTT
Stem SeqA	5738-40 CGG→GCC 5753-55 CCG→GGC	GGGCTTCTCCCCGGTTTTTTCCCGGCCCGGGGAGAAGTAGATTGAAG
Stem SeqB	5735-37 GGG→CCC 5756-58 CCC→GGG	GGGCTTCTGGGGCCTTTTTTCCCGGCGGCCCCAGAAGTAGATTGAAG
Stem SeqC	5732-34 AGA→TCT 5759-61 TCT→AGA	GGGCTGAGCCCGCCTTTTTTCCCGGCGGCGGGCTCAGTAGATTGAAG
Stem SeqD	5730-31 GA→CT 5762-63 TC→AG	GGGAGTCTCCCGCCTTTTTTCCCGGCGGCGGGAGACTTAGATTGAAG
Stem SeqE	5730-40 and 5753-63 Stem Seq A-D combined	GGGGAAGAGGGCGGTTTTTTCCCGGCCCGCCCTCTTCTAGATTGAAG
Mouse wt	mouse Wild-Type	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5162	5162 A→G	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGGTAGGAGTAGATTGGAAG
Mouse 5163	5163 G→A	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGGTAAAAGTAGATTGGAAG
Mouse 5164	5164 A→T	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGGTTGAAGTAGATTGGAAG
Mouse 5165A	5165 T→A	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGGAAGAAGTAGATTGGAAG
Mouse 5165B	5165 T→C	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGGCAGAAGTAGATTGGAAG
Mouse 5166	5166 G→A	AAGACTTCTACCGCCATTTTTTTTTTTCGGCGATAGAAGTAGATTGGAAG
Mouse 5168	5168 C→G	AAGACTTCTACCGCCATTTTTTTTTTTCGGGGGTAGAAGTAGATTGGAAG
Mouse 5169	5169 G→A	AAGACTTCTACCGCCATTTTTTTTTTTCGACGGTAGAAGTAGATTGGAAG
Mouse 5171	5171 C→T	AAGACTTCTACCGCCATTTTTTTTTTTGGGCGGTAGAAGTAGATTGGAAG
Mouse 5172	5172 T→C	AAGACTTCTACCGCCATTTTTTTTTTCCGGCGGTAGAAGTAGATTGGAAG
Mouse 5177	5177 T→C	AAGACTTCTACCGCCATTTTTCTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5178	5178 T→C	AAGACTTCTACCGCCATTTTCTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5190A	5190 T→C	AAGACTTCCACCGCCATTTTTTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5190B	5190 T→A	AAGACTTCAACCGCCATTTTTTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5194	5194 C→T	AAGATTTCTACCGCCATTTTTTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5198A	5198 A→T	TAGACTTCTACCGCCATTTTTTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Mouse 5198B	5198 A→G	GAGACTTCTACCGCCATTTTTTTTTTTCGGCGGTAGAAGTAGATTGGAAG
Loop+3nts	duplication 5747-49	GGGCTTCTCCCGCCTTTTTTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
Loop+2nt	duplication 5747-48	GGGCTTCTCCCGCCTTTTTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
Loop+1nt	duplication 5747	GGGCTTCTCCCGCCTTTTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
Loop-1nt	deletion 5746	GGGCTTCTCCCGCCTTTTTTCCGGCGGCGGGAGAAGTAGATTGAAGC
Loop-2nts	deletion 5745-46	GGGCTTCTCCCGCCTTTTTTCGGCGGCGGGAGAAGTAGATTGAAGCC
Loop-3nts	deletion 5744-46	GGGCTTCTCCCGCCTTTTTTGGCGGCGGGAGAAGTAGATTGAAGCCA
Mouse -3T	deletion 5181 and 5182	AAGACTTCTACCGCCATTTTTTTTCGGCGGTAGAAGTAGATT
Mouse -1T	deletion 5182	AAGACTTCTACCGCCATTTTTTTTTTCGGCGGTAGAAGTAGATT

Supplementary table S1. cont.

Name	Modification	Sequence 5' to 3'
Mouse +1T	duplication 5182	AAGACTTCTACCGCCATTTTTTTTTTTTCGGCGGTAGAAGTAGATT
Mouse +2T	duplication 5181and 5182	AAGACTTCTACCGCCATTTTTTTTTTTTTCGGCGGTAGAAGTAGATT
Mouse +3T	duplication 5180-82	AAGACTTCTACCGCCATTTTTTTTTTTTTTCGGCGGTAGAAGTAGATT
TTTTAA	5747-48 TT→AA	GGGCTTCTCCCGCCTTTTAACCCGGCGGCGGGAGAAGTAGATTGAAG
TTTAAA	5747-49 TTT→AAA	GGGCTTCTCCCGCCTTTAAACCCGGCGGCGGGAGAAGTAGATTGAAG
TTAAAA	5747-50 TTTT→AAAA	GGGCTTCTCCCGCCTTAAAACCCGGCGGCGGGAGAAGTAGATTGAAG
TAAAAA	5747-51 TTTTT→AAAAA	GGGCTTCTCCCGCCTAAAAACCCGGCGGCGGGAGAAGTAGATTGAAG
ATTTTT	5752 T→A	GGGCTTCTCCCGCCATTTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
AATTTT	5751-52 TT→AA	GGGCTTCTCCCGCCAATTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
AAATTT	5750-52 TTT→AAA	GGGCTTCTCCCGCCAAATTTCCCGGCGGCGGGAGAAGTAGATTGAAG
TATTTT	5751 T→ A	GGGCTTCTCCCGCCTATTTTCCCGGCGGCGGGAGAAGTAGATTGAAG
ATAAAA	5752 T→A; 5747-50 TTTT→AAAA	GGGCTTCTCCCGCCATAAAACCCGGCGGCGGGAGAAGTAGATTGAAG

Supplementary figure S1.

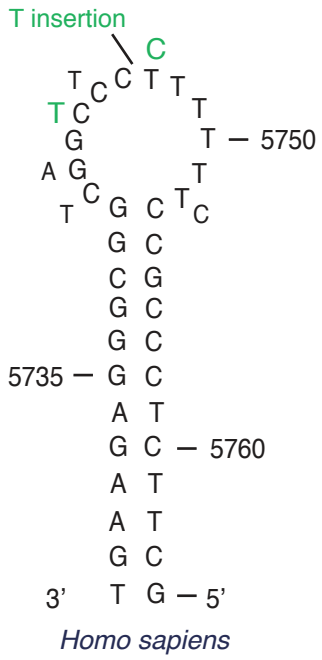

Figure S1. Naturally occuring sequence polymorphisms in human OriL.
Frequent changes are indicated in green.

Supplementary figure S2.

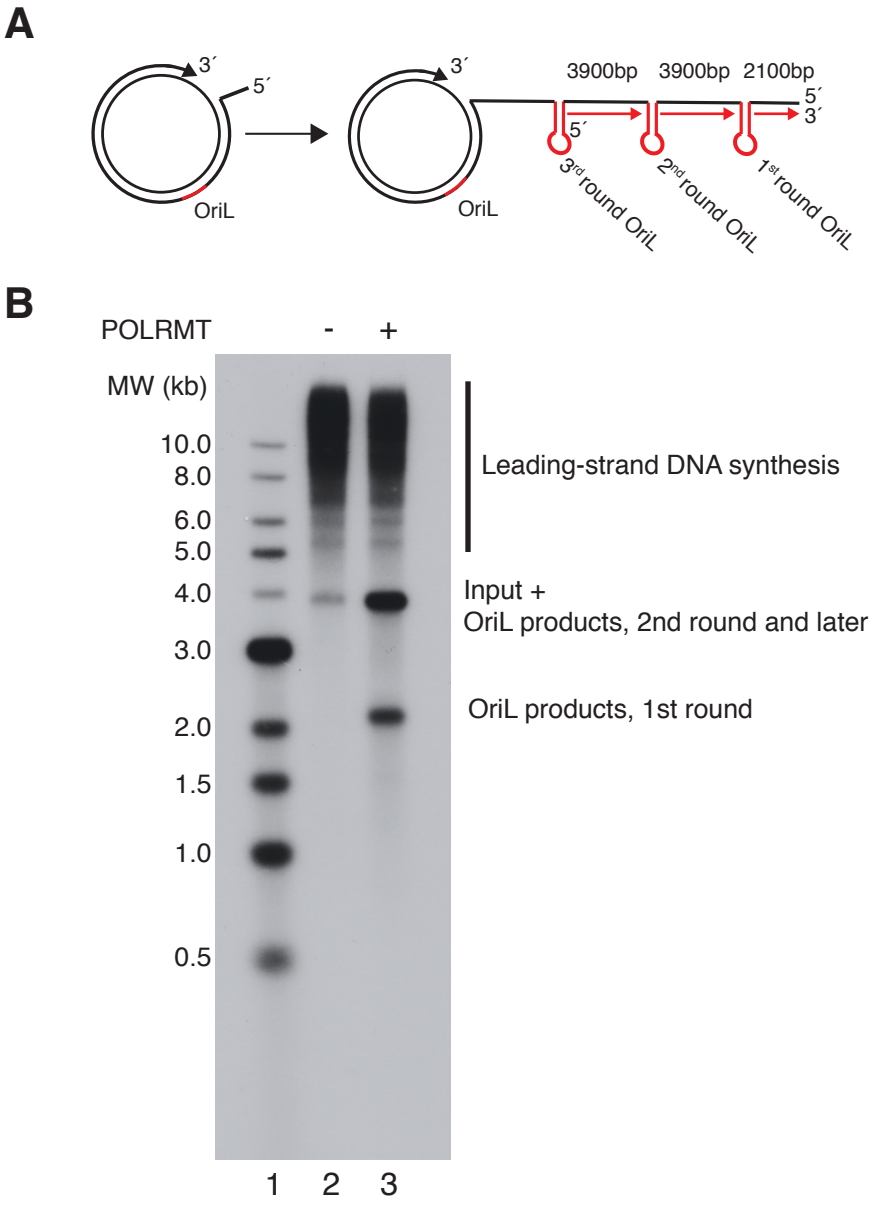

Figure S2. Products formed during rolling-circle mtDNA replication in vitro. (A) Schematic illustration explaining the replication products formed by OriL dependent initiation of lagging-strand DNA synthesis. During the first round of DNA synthesis, the OriL-depending lagging-strand products have a length of about 2100 nts, starting from OriL and stopping at the 5′-end of the template strand. This lagging-strand product can be easily observed, since it is clearly distinct in size from the input template. However, as leading-strand DNA synthesis progresses, OriL is exposed a 2nd, 3rd, and maybe even a 4th time. These later lagging-strand initiation events, will lead to full-length DNA products, i.e. these replication events will progress until they reach the 5′-end of the previous lagging-strand replication event at OriL. The fragments will therefore span the entire distance between two OriL sequences on the leading-strand DNA template (about 3900 nts). The length of these lagging-strand DNA synthesis products will thus be nearly identical to the labeled input template and impossible to separate from the input band, which thus appears stronger. **(B)** Separation of replication products on a denaturing agarose gel (0.7%, w/v). In the absence of POLRMT (lane 2) only leading strand products can be observed. Templates, not used for productive, rolling-circle replication are also labelled in this reaction. This labeling is most likely explained by POLγ idling on the free 3′-end of the input template.In the presence of POLRMT (lane 3), first passage OriL products migrate as a uniqe band of 2100 nts. Later rounds of OriL-dependent synthesis migrate with the same size as the input template. Therefore, the input band appears stronger, in reactions with active OriL-dependent DNA synthesis.

Supplementary figure S3

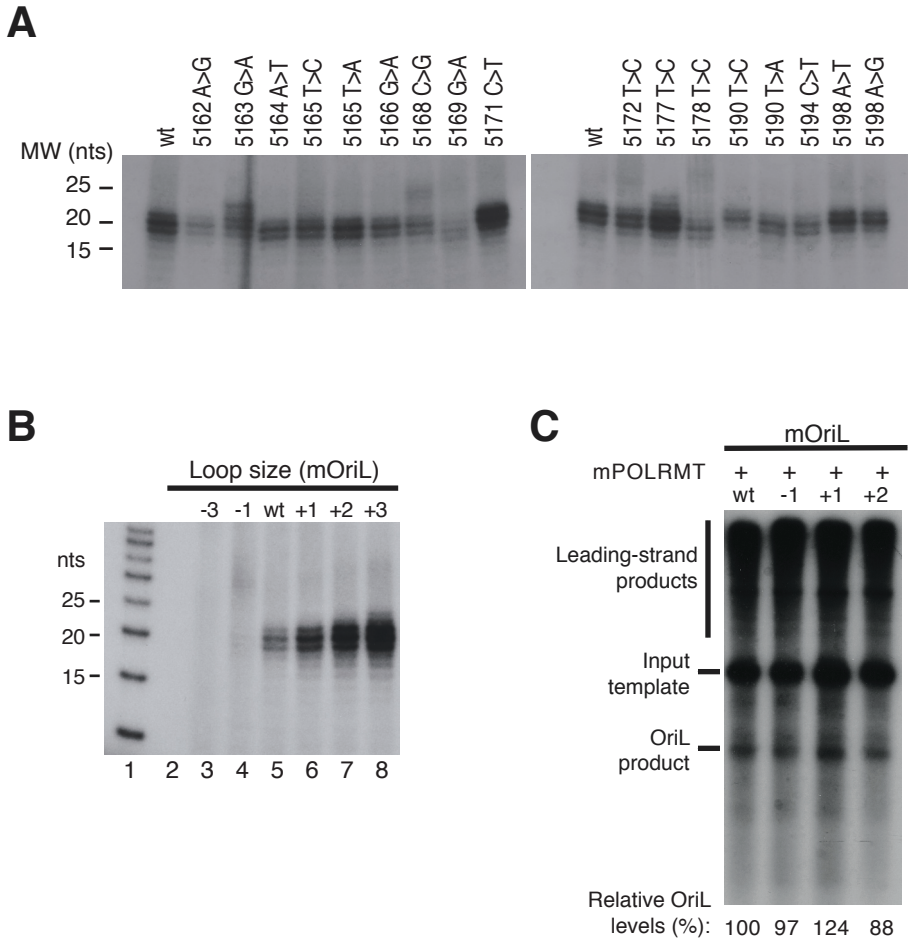

Figure S3. Primer synthesis and rolling-circle DNA replicatoin using recombinant mouse proteins. (A) All mutations identified in the stem-loop structure of mouse OriL (see Figure 1 C) could support primer synthesis in vitro, even if the efficiency varied somewhat between templates. Primase reactions were performed in the presence of mouse Polrmt and mtSSB on ssDNA mouse templates in the presence of [α- 32P]-GTP to label newly synthesized RNA. The sequence of the templates was modified as indicated and the exact sequences are provided in Table S1. After 30 minutes at 32 °C, reactions were analyzed on 25 % PAGE/Urea gels as described in experimental procedures. **(B)** Poly T-stretch (loop size) length variations found in mutator (polγAD257A/ polγAD257A) mice do not affect priming. Primase reactions were performed as in Figure 2 A but in the presence of mouse OriL (mOriL) and mouse Polrmt. The T-repeat length in mouse OriL was modified with between -3 to +3 nts, as indicated and the exact sequences are provided in Table S1. **(C)** In vitro lagging-strand replication efficiency is not influenced by the poly-T stretch length variations found in mutator (polγAD257A/ polγAD257A) mice. Activity of wt mouse OriL and indicated mutant derivatives was monitored as described in Figure 2 C, but in the presence of mouse Polrmt instead of human POLRMT.

Supplementary figure S4.

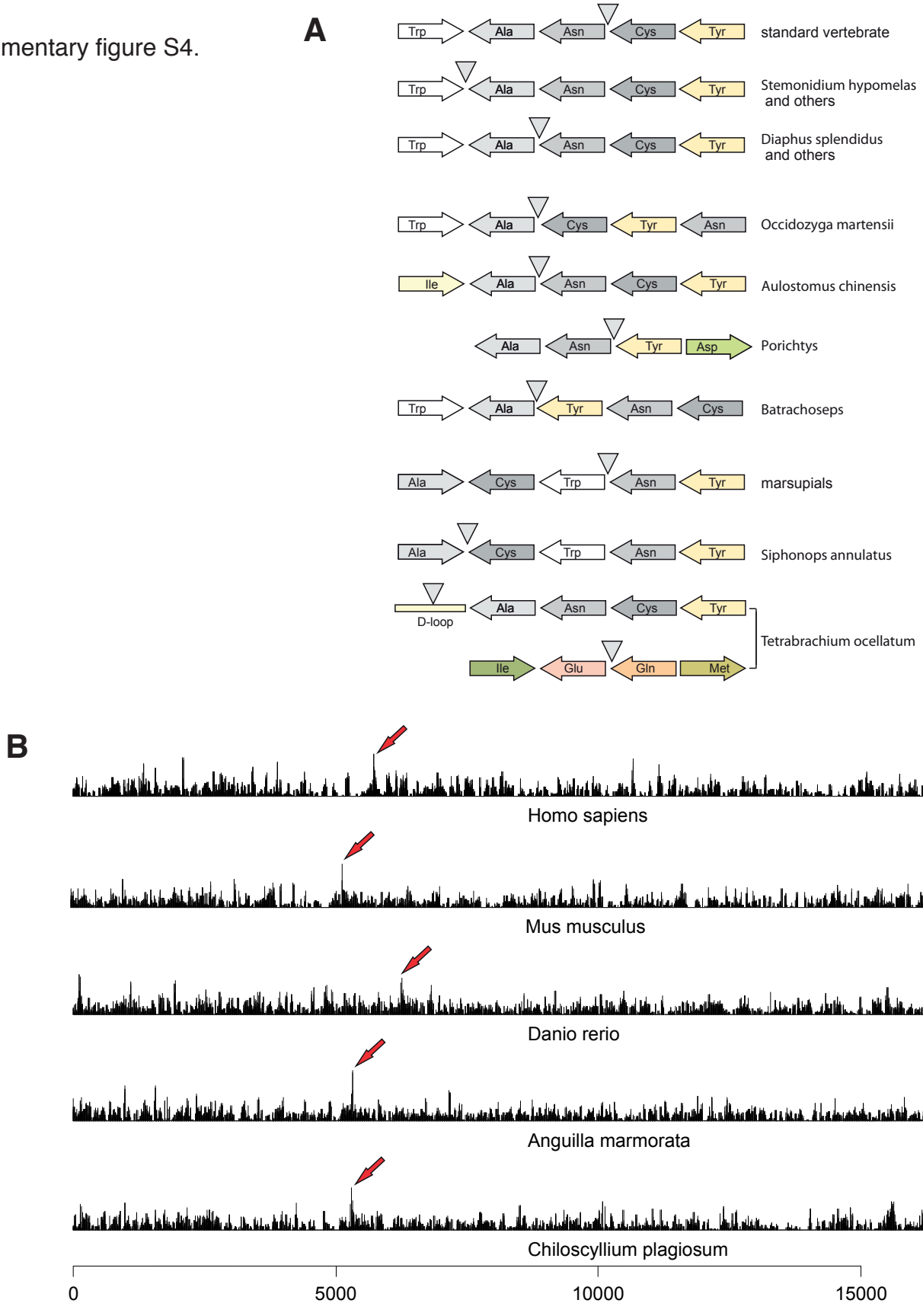

Figure S4 . (A) Recombination events involving OriL. A selection of representative genomes are displayed. (B) Hairpin structures was identified in mitochondrial genomes using UNAFold (Markham and Zuker, 2008). The OriL sequence forms a stable hairpin (red arrows).