SUPPLEMENTAL APPENDIX

Materials and Methods

Cell culture and mitochondrial DNA isolation

Human and mouse cells were cultured in DMEM, 1X penicillin/streptomycin with 10% FBS. Cells were harvested in PBS and mtDNA extracted as described previously (35). Briefly, cells were disrupted by homogenization in hypotonic buffer with a glass dounce homogenizer. After restoring to isotonic conditions mitochondria were purified by differential and sucrose-gradient centrifugation. After lysis of the mitochondria and proteinase K digestion, nucleic acid was isolated via phenol-chloroform extraction and aliquots of DNA were stored in isopropanol at -20°C.

Generation of *Rnaseh1* conditional knockout MEFs

MEFs were prepared from the trunks of 14.5 days old embryos using culture media (DMEM-high glucose, 15% FBS, 1X penicillin/streptomycin), as described previously (31). Both MEF cell lines (control and CKO-7) are homozygous for conditional *Rnaseh1*. CKO-7 is also homozygous for 4-hydroxytamoxifen inducible Rosa Cre. Both parents of these embryos had a genotype of homozygous conditional *Rnaseh1* and heterozygous tamoxifen inducible Rosa Cre The Jackson Laboratory - Gt(ROSA)26Sor$^{tm1(Cre/Esr1)Nat}$ mice (33) [MGI Ref ID J:84747]. Deletion of the *Rnaseh1* gene from MEF cells was achieved by induction of Cre recombinase by addition of 4-hydroxytamoxifen (4HT) at final concentrations of 100 nM (Sigma). Excision of *Rnaseh1* within 48 h was demonstrated via PCR with primers that flanked one of the loxP sites (Fig. S1B). DNA was isolated from purified mitochondria by successive phenol-chloroform extractions after detergent lysis and protease digestion, as previously (17), from MEFs treated for 5-12 days with 4HT.

Nucleic acid digestion, modification and analysis

Restriction digestions of 2 µg lots of purified mtDNA were performed in 200 µl volumes following the manufacturer's instructions, and then precipitated with ethanol and salt and resuspended in 1x TE buffer. Where indicated samples were treated additionally with 1 to 1.5 unit of *E. coli* RNase HI (Promega or Takara) for 15-30 min at 37°C. Single stranded nuclease (SSN) treatments employed 1U of mung bean nuclease (Takara) for 2 min at 37°C. RNase-free DNase (Ambion) treatments were 5U for 10 min at 37°C. DNA samples were denatured by mixing with an equal volume of formamide, heating to 95°C for 5 min and quenching on ice for 2 min. DNA was fractionated on a 2.35-2.5% Nu-Sieve 3:1 agarose (Lonza) gels with 0.1 mg/mL ethidium bromide, at 17.5 V/cm for 4-6 h at room temperature. Electrophoresis buffer was 10 mM sodium

borate (36), which was circulated continuously. After electrophoresis the gels were soaked for 30 min in 5 x SSC and 50 mM NaOH and transferred to solid support overnight by capillary transfer using 5 x SSC, 10 mM NaOH. Nucleic acids were UV cross-linked to the membrane by 1200 µJ/cm. Membranes were then probed with radiolabeled strand-specific RNA probes, using T7-maxiscript kit (Ambion) as per the manufacturer's instructions. Murine H and L-strand riboprobe templates were generated via PCR, using the primer pairs listed below.

Probing of the membrane (Magnaprobe) was performed overnight at 55°C in 2 x SSPE, 2% Sodium dodecyl sulfate, 5 x Denhardt's Reagent, 5% Dextran sulfate buffer. After overnight incubation, membranes were washed 4-6 times with 0.1 x SSPE, 0.5% SDS, at 55°C. Membranes were exposed to phosphorscreens (GE Healthcare) for 12-120 h and imaged on a Typhoon scanner (GE Healthcare). LM-PCR of mouse mtDNA was performed as described previously (7), using primers listed in SI appendix. Markers for Ori-H mapping utilized a forward primer with a 5´ end at nt 14,881 and a reverse primer with a 5´ end at LSP (16,183), CSB 1-3 or 16,034. The PCR products were digested with MscI prior to heating to 85^{o}C for 15 mins and formamide denaturation and 1D-AGE. An analogous approach was used for markers for mapping Ori-b ends and the RNA primer near LSP except that the restriction enzymes were EciI and HincII, respectively (primer sequences for the markers are listed below).

Primer pairs for hybridization probes (5´-3´):

H16200-200 TAATACGACTCACTATAGGAAATATGACTTATATTTTAGTACTTGTAAA
AGCTATTTTAATGTGCTTGATACC
H15450-16030 TAATACGACTCACTATAGGTACATTTATGTATATCGTACATT
TAGGTGATTGGGTTTTGCGG
H15511-16034 TAATACGACTCACTATAGGCAATGGTTCAGGTCATAAAATAATCATC
GCCTTAGGTGATTGGGTTTTGC
H15700-16,007 TAATACGACTCACTATAGGCGTGAAACCAACAACCCGCCCA
AATGATTCTTCACCGTAGGTGCG
H15007-15805 TAATACGACTCACTATAGGCTAGGAGGTGTCCTAGCCTTAATC
CGATAACGCATTTGATGGCCCTG
H15500-15750 TAATACGACTCACTATAGGAGCTTATATGCTTGGGGAAAATAG
GGGCCCG GAGCGAGAAGAGG
H14881-15490 TAATACGACTCACTATAGGTCCCAGACATACTAGGAGACCCA
CTTGGGGAAAATAGTTTAATGTACG
H14100-14400 TAATACGACTCACTATAGGTGACATGAAAAATCATCGTTGTAATT
CGTTTGCGTGTATATATCGGATT
H8031-8625 TAATACGACTCACTATAGGACGCCTAATCAACAACCGTCTCC
CATGGACTTGGATTAACTATGTGATATGC
L16200-200 AAATATGACTTATATTTTAGTACTTGTAAA
TAATACGA CTCACTATAGGAGCTATTTTAATGTGCTTGATACC
L15511-16183 CAATGGTTCAGGTCATAAAATAATCATC
TAATACGAC TCACTATAGGGATCAGGACATAGGGTTTGATAG

L14100-14400 TGACATGAAAAATCATCGTTGTAATT
TAATACGACT CACTATAGGCGTTTGCGTGTATATATCGGATT
L8124-8424 AACATGAACCCTAATAATTGTTTCCCTAAT
TAATACGAC TCACTATAGGTGCAGTAATGTTAGCTGTAAGC
L4872-5170 CTAAACCCAACCTAATATTT
GGCGGTAGAAGTAGATTGAA
L2922-3222 AAAGAACCAATACGCCCTTTAACAA
TAATACGACTCACTATAGGCATTTATTAATAGAACTGATAAAAGGATAA
L1912-2267 CGGCAAACAAGAACCCCGCC
TCTCCGAGGTCACCCC AACC
Underlined is the T7 promoter sequence

Primers to confirm RNase H1 gene excision

BC1417 5′-GAAGCACCTTAGCAGTATCTCCAGCAC-3′

BC1418 5′-GCTCCATCC TCAGTGGCACATAATC-3′.

Primers for Q-PCR of murine mtDNA

Nuclear DNA primers to mouse β-actin 5′-CAACAACAGTGCCCAATAGACA-3′ and 5′-GGAGAAAGCCTGGCAACATAG with probe 5′-CAGGAAGCAACTGGCAGAGAATTT GG-3′; and to mouse mtDNA COXII 5′-CCATCCCAGGCCGACTAAA-3′ and 5′-CAGA GCATTGGCCATAGAATAACC-3′ with probe 5′-CAAGCAACAGTAACATCAAACCGA CCA-3′.

Primers for Ligation-Mediated PCR

H15051-15072 5′-CCTAATACCTTTCCTTCATACC-3′

H15072-15098 5′-CTCAAAGCAACGAAGCCTAATATTCCG

H15939-15967 5′-CATCAACATAGCCGTCAAGGCATGAAAGG-3′

H15353-15377 5′-GACATCAAGAAGAAGGAGCTACTCC-3′

Unidirectional linkers:

LMPCR1 5′-GCGGTGACCCGGGAGATCTGTATTC-3′

LMPCR2 5′-GAATACAGATC-3′

Primers for Ori-H markers

L14881 5′-TCCCAGACATACTAGGAGACCCA-3′

H16034 5′-GCCTTAGGTGATTGGGTTTTGC-3′

H16052 5′-ATTGTTCGTACTTATTAATCGGA-3′

H16099 5′-ACCCCCCAAACCTCTCAATTTTA-3′

H16129 5′-AAAAACCCCAAACCGTAATTCTC-3′

H16183 5′-CTAGTCCTGTATCCCAAACTATC-3′

Primers for Ori-b markers

L14881 5′-TCCCAGACATACTAGGAGACCCA-3′

H15490 5′-GCTTGGGGAAAATAGTTTAATGT-3′

H15512 5′-GATTTAATGTACTAGCTTATATG-3′

H15527 5′-ATGACCTGAACCATTGATTTAAT-3′

H15625 5′-GTATGTCAGATAACACAGATATG3′

H15700 5′-GAGGATGGTAGATTAATAGACC-3′

For Figs. 2 and S3

Or,for Fig. S4

L14881 5′-TCCCAGACATACTAGGAGACCCA-3′

H15450 5′-CTGTTGTACTATGTAAATTTATGTACTC-3′

H15550 5′-GATTTATGTTGATGATTATTTTATGACC-3′

H15600 5′-TTATAACATTAGTTTAATGTGTTTAAG-3′

H15650 5′-GAGAAGAGTTTATGACTGTATGG-3′

H15700 5′-GAGGATGGTAGATTAATAGACC-3′

PCR products were digested with EciI to create a consistent end at np 15,084 prior to 1D-AGE fractionation of the markers.

Primers for L-strand LSP markers

H526 5′-TAGGTTTATGGCTAAGCATAGTGG-3′

L15950 5′-GGCCTACTTTCATCAACATAGC-3′

L16100 5′-ATTTTAACTCTCCAAACCCCCCA-3′

L16150 5′-AAAAACACTAAGAACTTGAAAGACA-3′

L16183 5′-AACTATCAAACCCTATGTCCTGAT-3′

L16225 5′-CAAAATATGACTTATATTTTAGTACT-3′

PCR products were digested with HincII to create a consistent end at np 366, prior to 1D-AGE fractionation of the markers.

PCR Conditions

50 ng of template DNA was incubated with 200 μM of each of the 4 dNTPs, 1.25 U of EX-Taq DNA Polymerase (Takara) in 50 μl of 1 X buffer supplied by the manufacturer, for 30 cycles of 94°C, 30 sec; Tm °C (lower of the 2 primers), 30 sec; 72°C, 30 sec. The 30 cycles were preceded by denaturation for 3min at 94°C, and followed by a 5 min incubation at 72°C.

Supporting Figures:

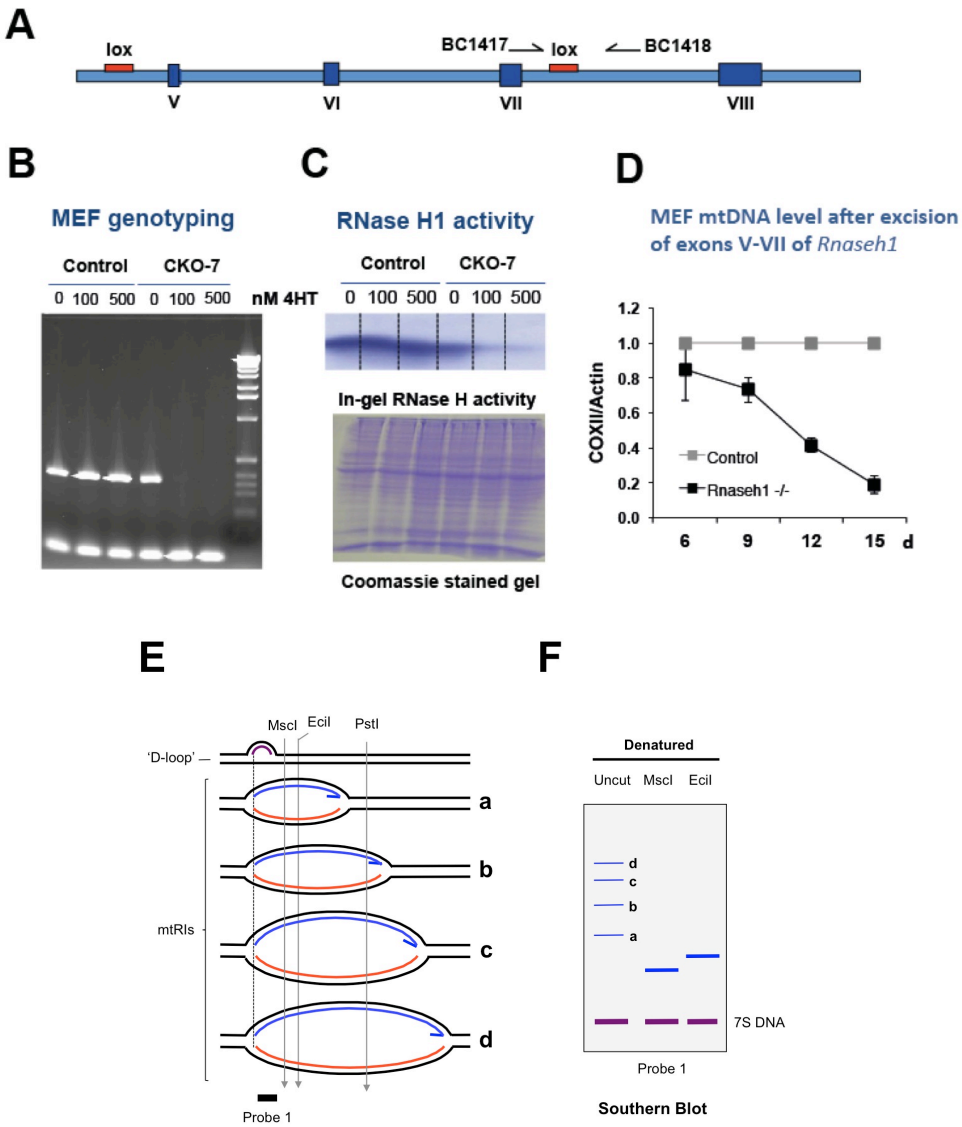

Figure S1. Generation of *Rnaseh1* conditional knockout MEFs and Mapping Strategy. A) *Rnaseh1* conditional knockout MEFs were generated by inserting LoxP sites (red) in the mouse nuclear genome at positions flanking exons (blue) V and VII of the *Rnaseh1* gene. **B**) The MEFs also carried a 4-hydroxytamoxifen (4HT) inducible cre-recombinase gene and addition of 4HT for final concentration of 100 or 500 nM to the culture medium led to the excision of the *Rnaseh1* based on PCR analysis with primers BC1417 and BC1418 that flank the exon VII-proximal loxP site, the fastest migrating band present in all samples is commensurate with primer-dimer. 4HT-treatment of the conditional knockout MEFs also led to **C**) the loss of RNase H1 activity evidenced by in-gel activity analysis, performed as previously described (33) and **D**) mtDNA depletion based on Q-PCR analysis of fragments of nuclear and mitochondrial DNA with primers to mouse β-actin and COXII of mtDNA (detailed elsewhere in the SI Appendix). **E**) The strategy for mapping nascent strands of mouse mtDNA is shown based on four representative intermediates (a-d) of the many that form during mtDNA replication. The illustration assumes that the 7S DNAs that form the D-loop and the nascent H-strands of replication intermediates share the same 5′ end of DNA, although the technique does not rely on this supposition, as 7S DNAs have the same size with the restriction enzymes used in this study, i.e. MscI, EciI and PstI for mouse mtDNA. **F**) A virtual Southern blot is illustrated showing the positions of 7S DNA and intermediates a-d on an agarose gel after denaturation and electrophoresis. Applying probe 1 to MscI digested mouse mtDNA will reveal nascent fragments of the same size, which will be slightly longer in the case of an EciI digest. Although species a-d all have a

variable length product after digestion it is not detected by the probe.

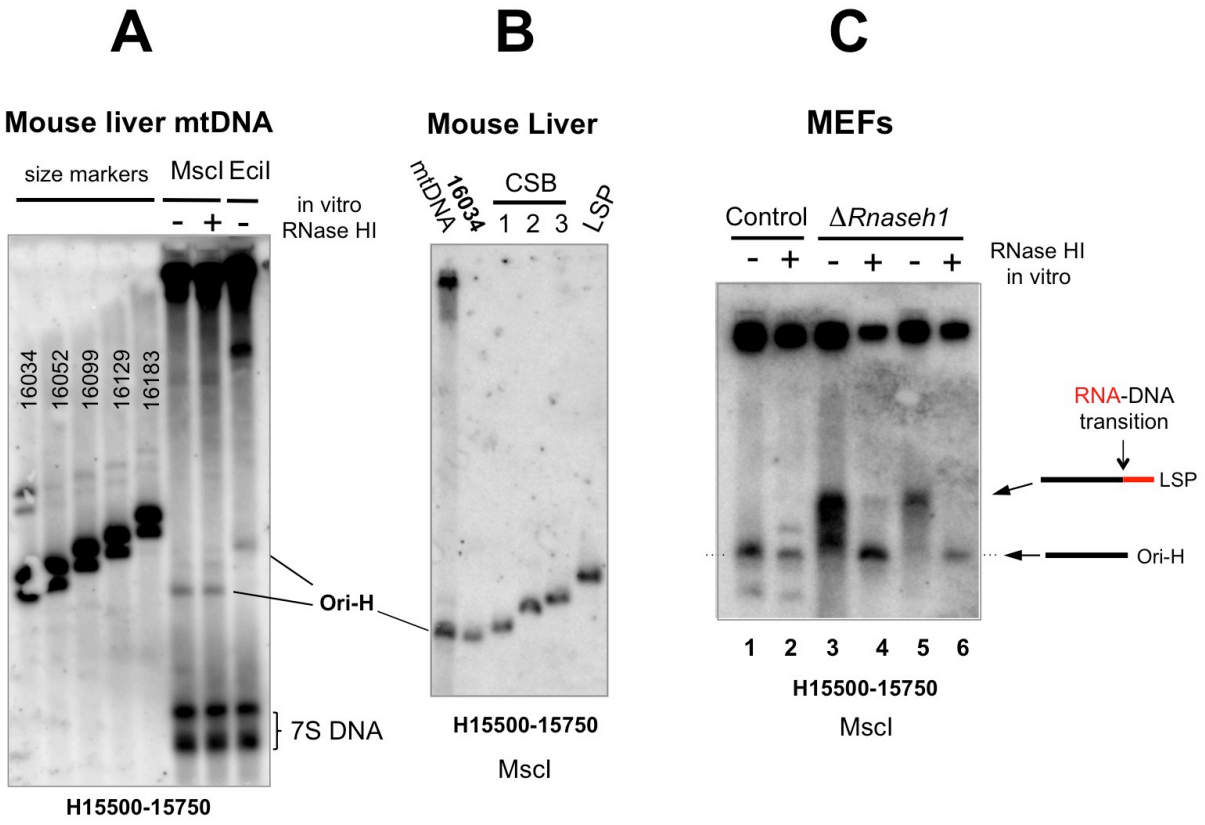

Figure S2. Mouse liver mtDNA samples contain nascent DNA strands with ends at Ori-H that lack a primer spanning LSP to Ori-H, and further examples of such a primer in MEFs lacking *Rnaseh1*. (**A**) and (**B**) Sodium borate 1D-AGE of denatured mouse liver mtDNA, after digestion with MscI or EcoI, and where indicated treated additionally (+) with Eco-RNase HI, reveals DNA ends mapping to Ori-H, and 7S DNAs that are not cut by these restriction enzymes. After 1D-AGE and blot transfer the mtDNA was hybridized to a riboprobe spanning nps 15,520-15,750 of the murine mitochondrial genome. As with the control MEFs (Fig. 1C, and panel **C**, lanes 1 and 2 of this figure), the mobility of the nascent strands of mouse liver mtDNA were not discernibly affected by Eco-RNase HI (RH), in striking contrast to MEFs lacking the *Rnaseh1* gene for 9 days (panel **C** lanes 3-6). Although the resolution limit of the sodium borate gels is 10-20 nucleotides and does not exclude the possibility of a short primer, there is no evidence of such a species in control mouse liver samples based on previous LM-PCR and primer extension studies (3, 7). On the other hand, a short primer of 20 or fewer ribonucleotides might explain the small fraction of RNase H-resistant material with an end at ~ 16,183 (LSP).

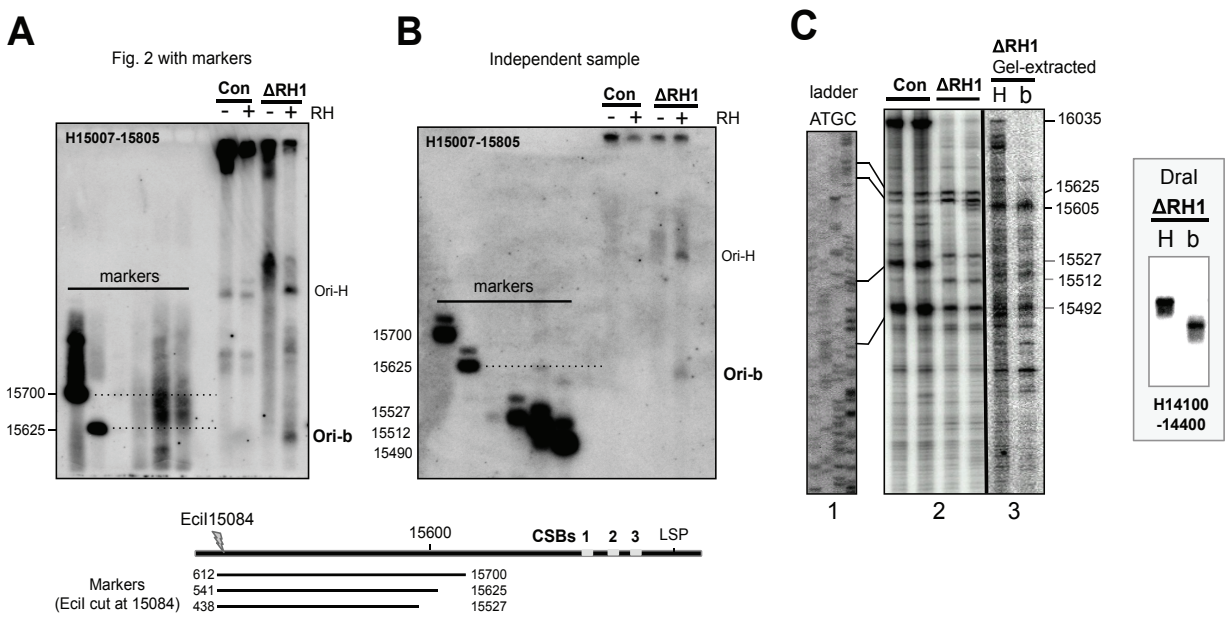

Figure S3. *Rnaseh1* ablation leads to the accumulation of free 5´ ends of DNA mapping to np ~15,605. (**A**) A reproduction of Figure 2, showing the EcilI digested PCR products that served as markers of defined lengths, as illustrated beneath the gel image (primer sequences are detailed elsewhere in the SI Appendix) and (**B**) an equivalent experiment with different mtDNA samples run for 5 h rather than 6 h, at 11V/cm. EcilI digested and denatured MEF mtDNAs were fractionated by 1D-AGE and blot hybridized to the probe H15007-15805. Samples were treated with (+), or without (-) Eco-RNase HI (RH), prior to denaturation. (**C**) LM-PCR products from a gel-excised truncated fragment mapping to Ori-b extend as far as np 15,605. LM-PCR of total mtDNA and gel excised DraI fragments of mtDNA derived from MEFs treated without (control) or with (ΔRH1) 4HT (see SI Appendix for details). The DNA sequencing ladder was prepared with an H-strand primer spanning np 111-130, using a plasmid containing the cloned human NCR region with the SequiTherm EXCELTM II DNA Sequencing Kit from Epicentre® Biotechnologies. Samples were fractionated by 6% SDS-PAGE. The end assigned as np 16,035 is an inference based on comparison with other gels (e.g. (7)). Inset: Southern blot analysis of gel-extracted DraI fragments of mtDNA spanning nt 14,178 to ~Ori-b or ~Ori-H, detected with probe H14100-14400.

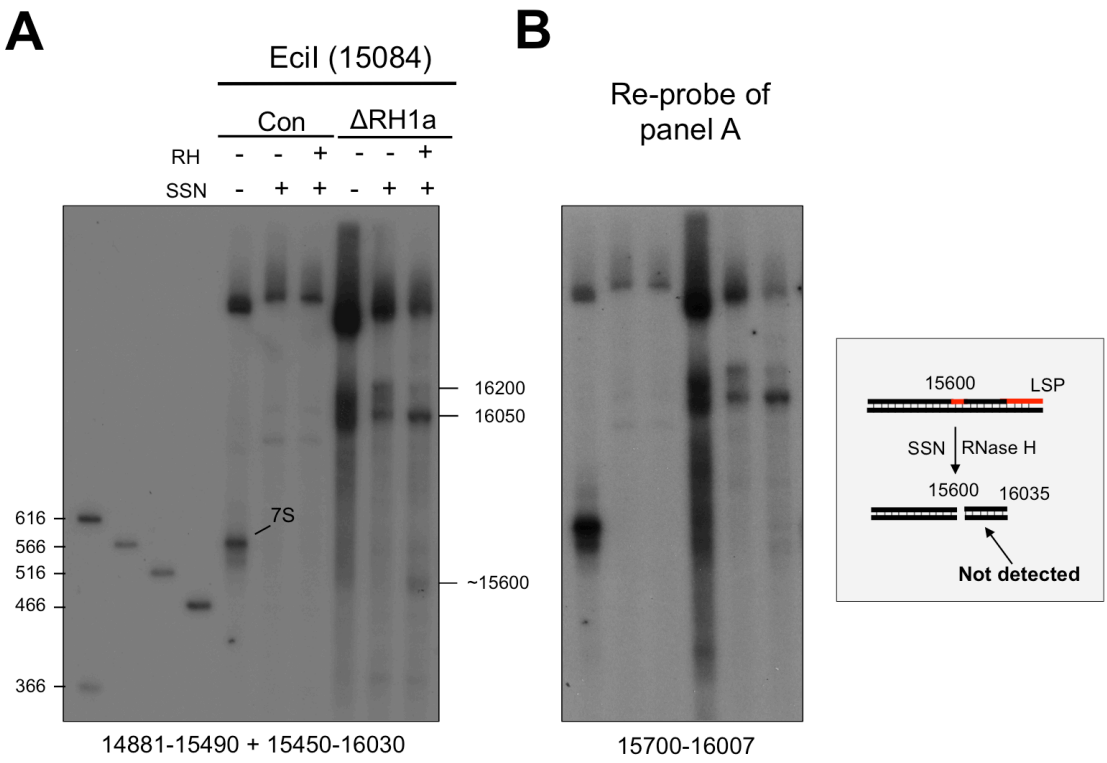

Figure S4. Absence of H-strand DNA between Ori-H and Ori-b on nascent strands initiated at Ori-b. The product of RNase HI treatment mapping to Ori-b (Figs. 2 and 3) could result from a priming event nearby, independent of that at LSP for Ori-H, as illustrated in the box. However, whilst a combined Eco-RNase HI (RH) and SSN treatment (after EciI digestion) once again yielded DNA ends mapping to Ori-b (**A**), there was no detectable DNA spanning the region between Ori-H and Ori-b (**B**). The absence of this piece of DNA supports the interpretation that both the Ori-b and the Ori-H primers initiate at LSP (as illustrated in Figs. 2 and 3). Samples were treated as per Fig. 3 and hybridized to probes spanning the indicated nucleotide numbers of mouse mtDNA. The primers and PCR conditions used to generate the markers are detailed elsewhere in SI appendix.

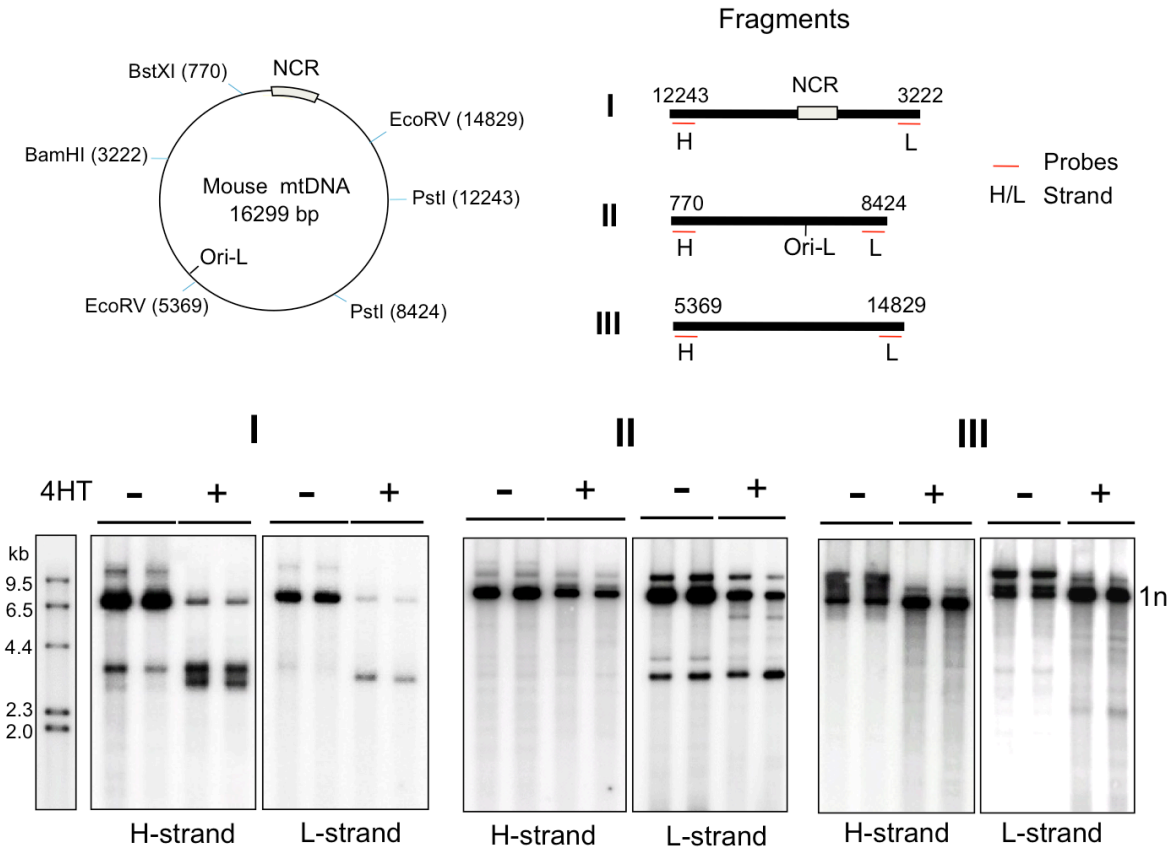

Figure S5. Genome-wide screen for prominent RNA patches in mtDNA of MEFs lacking RNase H1. Mouse mtDNA was restriction digested to produce three over-lapping (1n) fragments, I, II and III. Each fragment was probed at the 3′ end of the H- or L- strand (red line). Samples were digested, treated with nuclease, denatured in 80% formamide with heat, and separated by 1D-AGE. Size markers are [γ-32P] end-labeled λ-HindIII ladder. 4HT, 4-hydroxytamoxifen. Samples derived from MEFS retaining *Rnaseh1* 4HT-, whereas MEFs lacking *Rnaseh1* had been incubated with the drug for 9 days (4HT+).

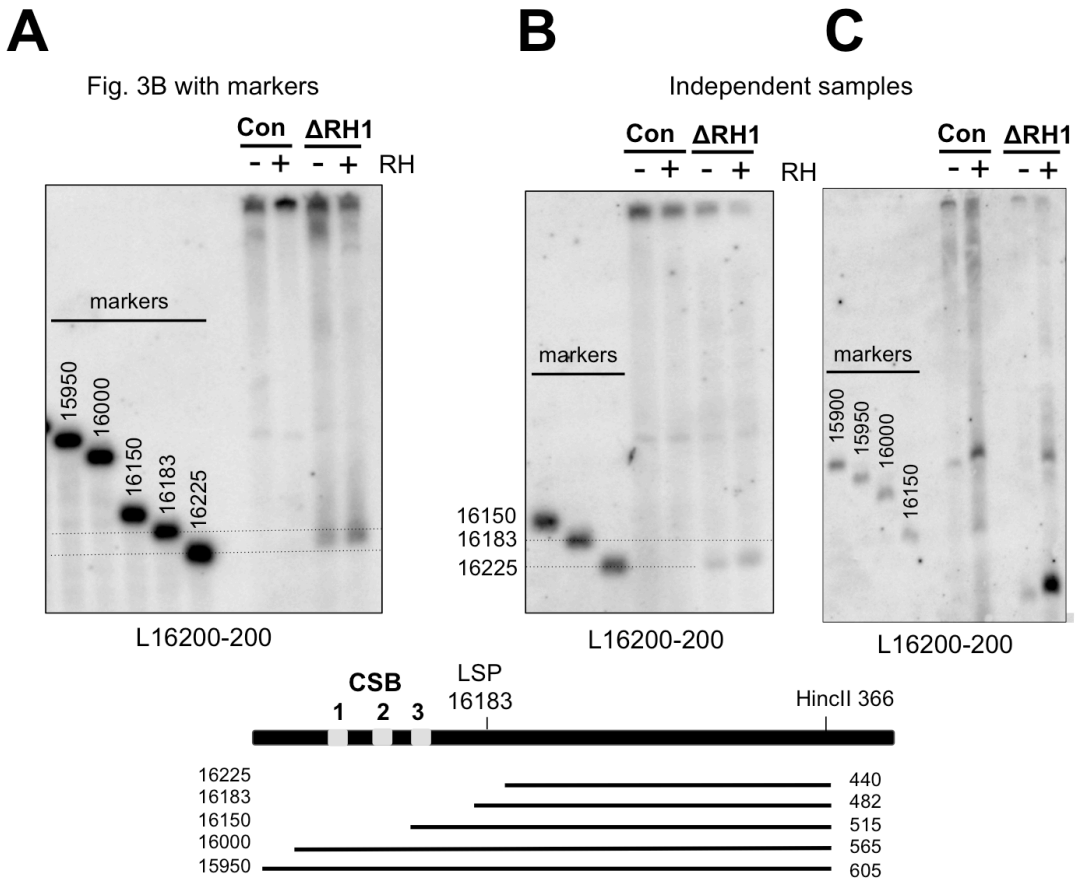

Figure S6. Mapping of an RNA patch on the L-strand close to LSP arising as a result of loss of *Rnaseh1*. (**A**) A shorter exposure of Figure 5B, additionally showing the synthetic DNAs of defined lengths, that served as markers, as illustrated beneath the gel images (primer sequences are detailed elsewhere in the SI Appendix) MEF mtDNA from cells culture for 8 (**A**), or 10 (**B, C**) days with 4HT (ΔRH1), or from MEFs cultured without the drug, was digested with HincII, denatured for 15 min at 85^{o}C, in 80% formamide and fractionated by 1D-AGE for 6 h at 11V/cm (2.3% (**A,B**), 2.75% (**C**) Sodium borate gels) and blot hybridized to probe L16200-200.

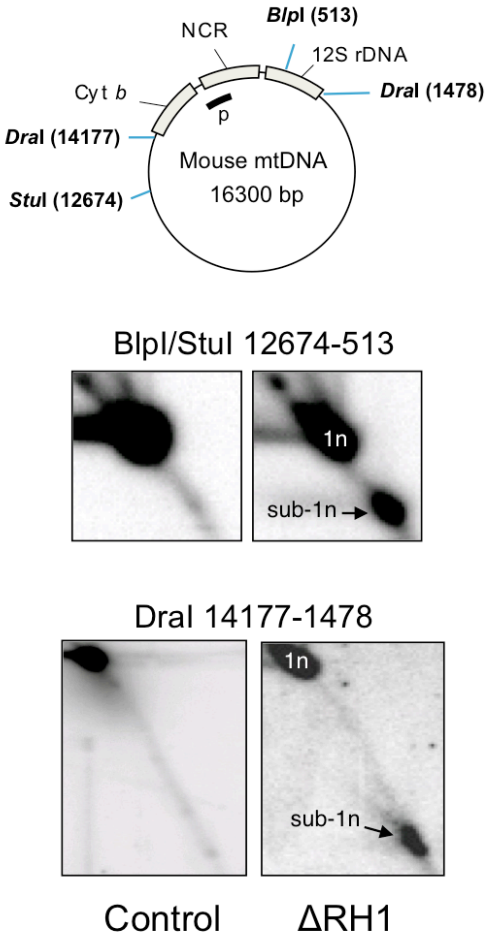

Figure S7. Abundant truncated fragments of mtDNA of MEFs after ablation of *Rnaseh1*. MEFs were cultured for 9 days with 4HT (ΔRH1) prior to mtDNA purification, restriction digestion and 2D-AGE. The truncated fragments (sub-1n) are compatible with initiation of replication on molecules containing a gap at the origin, owing to failure to complete the preceding round of replication (as illustrated in Fig. 7f).

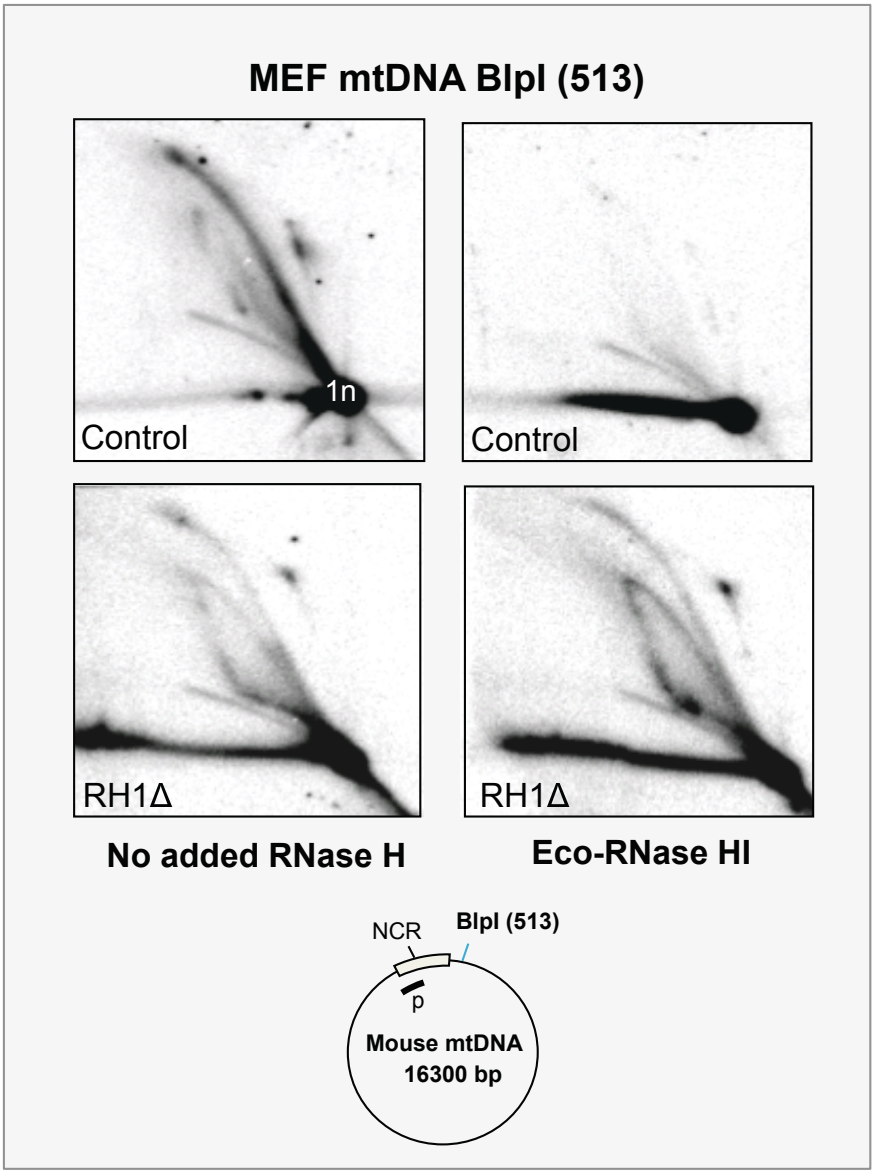

Figure S8. Ablation of *Rnaseh1* results in the formation of mitochondrial replication intermediates lacking the lengthy RNA/DNA hybrids associated with the bootlace mechanism of mtDNA replication. Mitochondrial DNA was harvested from conditional knockout MEFs cultured for 9 days without (control) or with 4HT (RH1Δ). BlpI digested MEF mtDNAs were fractionated by 2D-AGE as previously described (34), transferred to solid support and hybridized to a dsDNA probe spanning nt 15,338-16,034 of the murine mitochondrial genome.