**Supporting Information**

## Materials and Methods

**Patients:** Exome sequencing identified homozygous missense mutations in *RNASEH1*, c.424G>A; p.Val142Ile (V142I), in the affected members of two unrelated families with neuromuscular disease. All unaffected members of the families had at least one normal allele. In Family 1, the two affected siblings developed ptosis, chronic progressive external ophthalmoplegia, ataxia, proximal myopathy and a sensorimotor neuropathy in adult life; a very similar clinical phenotype was exhibited by the four affected siblings of Family 2. The muscle tissue of all affected individuals in both families manifested multiple mtDNA deletions and frequent cytochrome *c* oxidase and ragged red fibers (Fig. S5). The patient derived fibroblasts used in the study were from family 2 member III-9 (Fig. S5A).

**Mitochondrial DNA preparation from solid tissue:** Ten grams of finely chopped mouse liver was suspended in 9 vol homogenization buffer (HB)/g liver and subjected to 3 strokes of Dounce homogenization with a tight fitting pestle. HB is 225 mM Mannitol, 75 mM Sucrose, 2 mM EDTA and 10 mM HEPES-NaOH (pH 7.6). The homogenate was centrifuged at 600 $g_{max}$ for 10 min, and the supernatant re-centrifuged at 7,000 $g_{max}$ for 10 min to obtain a crude mitochondrial pellet. The mitochondrial pellet was re-suspended in 5 vol HB/g liver, centrifuged at 7,000 $g_{max}$ for 10 min, and this step was repeated with 2.5 vol HB/g liver. The second mitochondrial pellet was re-suspended in 0.5 vol HB/g liver and loaded on to a 1M/1.5M sucrose step-gradient, in 2 mM EDTA and 10 mM HEPES-NaOH (pH 7.8), and centrifuged at 40,000 $g_{max}$ for 1 h. The mitochondrial layer was diluted to 250 mM sucrose, 2 mM EDTA and 10 mM HEPES pH 7.8 and sedimented by centrifugation at 9,850 $g_{max}$ for 10 min. The mitochondrial pellet was re-suspended in 75 mM NaCl, 50 mM EDTA, 20 mM HEPES pH 7.2 and divided into 4 equal fractions (of ~10 mg) (a-d, see Fig. S2). All the above procedures were performed on ice in a cold-room. Mitochondria were incubated with 100 μg/ml Proteinase K on ice for either 45 min (A-C) or 10 min (D). Next the mitochondria were lysed with 1% sodium N-lauroylsarcosinate (sarcosine) and incubated at 50$^{o}$C for 45 min (a) 10 min (b), or 0 min (c and d) (Fig. S2); and the nucleic acids isolated by successive phenol and chloroform extractions, followed by 2-propanol precipitation. All chemicals used were RNase- and DNase-free grade. For

all the subsequent R-loop analysis the 50$^{o}$C step was omitted and Proteinase K treatments were performed on ice for 10-45 mins.

**Cell culture and mitochondrial DNA isolation:** Proliferating human cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), with 10% fetal bovine serum. Cells were harvested in PBS and mtDNA extracted as previously (1). For whole cell DNA isolation, cells were lysed in HB. After cell or mitochondrial lysis with 0.25% sodium sarcosine and digestion with 1 mg proteinase K per 5 x $10^6$ cells for 1-6 h at 37$^{o}$C, nucleic acids were isolated via phenol-chloroform extraction, and precipitated with 0.1 vol of 3M sodium acetate and 0.8 vol 2-propanol; DNA was recovered by centrifugation, re-suspended in 10 mM Tris pH 8.0 and stored at -20°C. For nucleic acids cross-linking, cells were pre-incubated in 10 μM 4,5',8-trimethylpsoralen (TMP) for 10 min at 37$^{o}$C and exposed to 365 nm UV light for a further 10 min at room temperature. The cells were washed once in PBS and lysed immediately to extract DNA as above. Quiescence was induced in the fibroblasts by reducing the serum concentration of the growth medium from 10% to 0.1% and the cells analyzed 10 days later.

**Nucleic acid digestion, modification and analysis:** Restriction digestions of 2-10 μg of whole cellular DNA, or 2-4 μg lots of DNA from purified mitochondria, were performed in 40-200 μl volumes according to the manufacturer's instructions, and then precipitated with ethanol and salt and re-suspended in 10 mM Tris pH 8.0. Where indicated samples were treated additionally with 1 U of E. coli RNase HI (Promega or Takara) for 15-60 min at 37°C. Incubation with recombinant SSB from E. coli (Promega) was as previously (2). Denaturation of DNA was achieved 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 in one dimension (1D-AGE) either on 0.8-1.5% Tris-acetate agarose (Invitrogen) gels overnight at 0.8 V/cm or 2-2.5% Nu-Sieve 3:1 agarose (Lonza) gels (10 mM sodium borate), at 17.5 V/cm for 4-6 h at room temperature with circulation. Two dimensional agarose gel electrophoresis was as previously described (3). After electrophoresis, gels were soaked for 30 min in 5 x SSC and 10 mM NaOH and then transferred to nylon membrane overnight by capillary transfer using 5 x SSC, 10-100 mM NaOH. DNA was cross-linked to the membrane by exposure to UV at 1200 μJ/cm. Membranes were then probed with radiolabeled DNA, prepared using a random-prime labeling kit (GE Healthcare); or riboprobes, using T7-

maxiscript kit (Ambion) as per the manufacturer's instructions. Probing of the membrane was performed overnight at 55-60°C in 2 x SSPE, 2% Sodium dodecyl sulfate, 5 x Denhardts Reagent, 5% Dextran sulfate buffer. After overnight incubation, membranes were washed four to six times with 0.1-1 x SSPE, 0.5% SDS, at 55°C. Membranes were exposed to X-ray film or phosphorscreens for 4-120 h and imaged on a Typhoon scanner (GE Healthcare). Image J software was used to quantify D-loop and R-loop levels.

**Detection and characterization of the mitochondrial R-loop:** To determine the composition of small bubble-like structures associated with fragments of mtDNA containing the control region, BclI digested mouse liver mtDNA was fractionated by 2D-AGE and the portion of the gel running slightly above the linear 4.15 kb fragment (np 12,034-16,179) was excised and the nucleic acids recovered by electrophoresis into dialysis tubing. Some gel-extracted material was treated with RNase HI, DNase or no enzyme, prior to denaturation and re-fractionated by 1D-AGE, as previously applied to mtDNA replication intermediates (3). The products were re-fractionated by 1D-AGE and hybridized to H- and L-strand specific riboprobes generated from amplified mouse (m) mtDNA with the following primers (5′-3′, with the T7 promoter sequence underlined.): TAATACGACTCACTATAGGCAATGGTTCAGGTCATAAAA TA ATCATC, m-H15,511-15,539 and GCCTTAGGTGATTGGGTTTTGC m-H16,034-16,012; CAATGGTTCAGG TCATAAAATAATCATC, m-L15,511-15,539 and TAATA CGACTCACTATAGGGCCTTAGGTGATTGGGTTTTGC m-L16,034-16,012.

For sequencing, gel-extracted material, or purified mtDNA was treated with 2 U Turbo DNase I (Ambion) in 20 µl at 37°C for 1 h; incubated with 20 U T4 RNA ligase in 1 x RNA ligase buffer, 12% PEG 6000 to circularize the RNA. For reverse transcription the RNA was incubated with 0.5 mM dNTPs, 0.5 µM primer R1 (m-L15,897-15,918 GTGGTGTCATGC ATTTGGTATC), 4 U Omniscript reverse transcriptase in 1 x RT buffer, in 20 µl at 37°C for 1 h. For PCR across the ligation junctions to identify the ends of the RNAs, the DNA was amplified using 1U Vent polymerase in 50 µl of 1 x amplification buffer, 0.1 µM primer F1 (m-H15,840-15,821 GGGAACGTATG GGCGATAAC) and R1, 0.25 mM dNTPs for 35 cycles of 95°C 2 min, 56°C 1 min, 76°C 1 min, and an elongation step of 10 min at 76°C. 2-5 µl of the PCR reaction served as template in a second amplification reaction comprising template, 3U BioTaq, 1 x BioTaq buffer, 1.5 mM $MgCl_2$, 0.12 µM primers F2 (m-H15,704-15,680

CACGGAGGATGGTAGATTAATAGA) and R2 (mH-15,949-15,969 GCCGTCAAG GCATGAAAGGAC), 0.3 mM dNTPs in 50 µl. Reaction conditions were 95°C for 3 min followed by 35 cycles of 95°C 0.5 min, 58°C 0.5 min, 72°C 0.5 min, and an extension step of 72°C for 10 min. The products were gel-extracted (QIAquick Gel Extraction Kit) and cloned into PCR2.1 vector using 50 ng of vector, 4U T4 DNA ligase in 1 x DNA ligase buffer for 14 h at 14°C. The ligated DNA was transformed into Top10 E. coli and clones carrying a DNA insert sequenced commercially. On one occasion the same procedure was applied to Trizol™ purified RNA from purified mouse liver mitochondria; and the ends of 7S DNAs were determined using the same primers and purified mtDNA, omitting the DNase treatment and reverse transcription steps.

Additional Primer pairs for hybridization probes:

**Mouse**

m-14903-15401: 5´-CAGACAACTACATACCAGCTAATCCAC and 5´-ACCAGCTTTG GGTGCTGGTG. m-15511-16034: 5´-ATCAATGGTTCAGGTCATAAAATAATCATCA AC-3´ and 5´-GCCTTAGGTGATTGGGTTTTGC-3´. m-H16051-16183: 5´-TAATAC GACTCACTATAGGGTTAGACATAAATGCTACTCAATACC-3´ and 5´-GATCAGGAC ATAGGGTTTGATAG-3´.

**Human (h)**

h-H15,869-168: 5´-TAATACGACTCACTATAGGAAAATACTCAAATGGGCCTGTCC-3´ and 5´-GGTGCGATAAATAATAGGATGAGG-3´. h-16,341-151: 5´-TTACAGTCAA ATCCCTTCTCGTCC-3´ and 5´-GGATGAGGCAGGAATCAAAGACAG-3´.

**Q-PCR of human mtDNA:** MtDNA was quantified as described in (4). Briefly, total DNA was isolated from human fibroblasts using DNeasy Blood and tissues Kit (QIAGEN). 25 ng DNA were used as template for amplification of COXII gene in a real-time quantitative PCR reaction (7550 Fast Real-Time PCR, Applied Biosystem). A portion of APP1 gene was amplified simultaneously as nuclear reference. Primers: hCOXII: 5´-CGTCTGAACTATCCTGCCCG-3´ and 5´-TGGTAAGGGAGGGATCGT TG-3´, hAPP-F: 5´-TTTTTGTGTGCTCTCCCAGGTCT, and 5´-TGGTCACTGGTTGG TTGGC-3´.

**Immuno-blotting:** Protein fractionation, transfer and immuno-detection were performed as described (5), with some modifications. Cells were lysed on ice in PBS, 1% SDS, 1 X protease inhibitors cocktail (Roche) and 50 U Benzonase (Millipore). Protein concentration was measured by Lowry assay (DC™ Reagent, Biorad) and 20 µg of lysates analyzed per lane. For RNAse H1 immunodetection, samples were treated with 2 mM DTT as reducing agent. Primary antibodies employed were: mouse anti-RNase H1 (abcam # 56560, 1:500) and mouse anti-VCL (abcam # 18058, 1:5000).

**Primers for markers of defined length:** for high-resolution 1D-AGE were generated by PCR amplification of human mtDNA with forward primer: h15869 5´-AAA ATACTCAAATGGGCCTGTCC-3´ and the following series of reverse primers: h-54 5´-CCAAATGCATGGAGAGCTCC-3´; h-111 5´-TGCTCCGGCTCCAGCGTC-3´; h-150 5´-GATGAGGCAGGAATCAAAGACA-3´; h-168 5´-GGTGCGATAAATAATAGG ATGAGG-3´; h-191 5'-TGTTCGCCTGTAATATTGAACG-3´; h-240 5'-TATTA TTATGTCCTACAAGCAT-3´; h-300 5'-TGGTGGAAATTTTTTGTTATGATG-3'; h-407 5'-AAAGATAAAATTTGAAATCTGGT-3'. PCR products were digested with Dra1 to create a consistent end at np 16,010 prior to 1D-AGE fractionation of the markers. Another marker spanning the BsaW1 site at np 15,924 to 50 was generated by digesting 143B crude mtDNA with BsaW1 and BstX1 to create a product of 699 bases in length (see Fig 5C).

**PCR Conditions:** Unless specified elsewhere in the methods, 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 reaction buffer (Takara), 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 3 min at 94°C, and followed by a 5 min incubation at 72°C.

**Northern Hybridization** RNA was extracted from fibroblasts using Trizol according to the manufacturers instructions. Three microgram aliquots were fractionated on 1% agarose gels in 1 x MOPS buffer, and blot hybridized to radiolabeled probes derived from regions of mtDNA amplified with primers 5'-CACCCAACAATGACTAATC AAACTAACCTC-3´ and 5´-TATGAGGAGCGTTATGGAGTGGAAG-3´ (A6/A8); 5´-TATTCCTAGAACCAGGCGACCTGC-3´ and 5´-TTTCGTTCATTTTGGTTCTCAGGG

TTTG-3´ (COX2); and 5´-CTGCCATCAAGTATTTCCTCACGC-3´ and 5´-TCAGGTGCGAGATAGTAGTAGGGTC-3 (ND2).

**Gene-silencing of RNase H1**: RNA interference of RNase H1 in human 143B osteosarcoma cells was as described (6). Briefly, 2 x $10^6$ 143B cells were transfected with lipofectamine RNAiMAX (Invitrogen) combined with 10 nM dsRNA [5´-GGAUGGAGAUGGACAUGAAAG-3´, 5´-UUCAUGUCCAUCUCCAUCCAG-3´] according to the manufacturer's instructions. The same number of cells was re-transfected 72 h after the first transfection and the nucleic acids harvested 72 h later. Repression of the target protein after siRNA was confirmed by immunoblotting.

**Immunocytochemistry**: Patient and control fibroblast cultures, the cells were incubated with 20 µM BrdU for 10 h and fixed with 2% paraformaldehyde 15 min at room temperature, treated with phosphate buffered saline (PBS) containing 0.2% Triton X-100. After a 5 min PBS wash, cells were incubated for 90 min at 40℃ in 2N hydrochloric acid (HCl). 5% goat serum in PBS was applied for 1 h, for blocking, after which the cells were incubated with primary antibody in PBS at 4℃ overnight and subsequently with secondary antibodies for 2 h at ambient temperature. A 90 min incubation at room temperature with Alexa Fluor 488 conjugated streptavidin (Invitrogen) was followed by three PBS washes. The coverslips were mounted on glass slides using Progold with DAPI. For staining which did not include BrdU the HCl antigen retrieval step was omitted. Primary antibodies: mouse anti-DNA Progen (AC-30-10) at 1:200; rat anti-BrdU Bio-Rad (MCA2060) 1:200; rabbit anti-MRPL45 (Proteintech) 1:200; rabbit anti-MRPS18b (Proteintech) 1:200; rabbit anti Tom20 (Santa Cruz) 1:400. Secondary antibodies: Alexafluor 488 goat anti-mouse (1:500); Alexafluor 488 goat anti-rat (1:500); Alexafluor 568 goat anti-rabbit (1:1000).

**[$^{35}$S]-methionine labeling of mitochondrial proteins**: mitochondrial translation products in cultured cells were labeled as described previously (5). Fibroblasts were washed twice with methionine/cysteine-free DMEM (Sigma) supplemented with 2 mM L-glutamine, 96 µg/ml cysteine and 5% (v/v) dialyzed FBS followed by a 10 min incubation in this medium at 37$^o$C. Cytosolic translation was inhibited by incubating the cells for 20 min with 100 µg/ml emetine dihydrochloride (Sigma) at 37$^o$C. 100 µCi [$^{35}$S]-methionine was added to the medium and the cells incubated for 1 h at 37$^o$C, washed three times with PBS and lysed in 1 X PBS, 0.1% n-dodecyl-D-maltoside

(DDM), 1% SDS, 50 U benzonase (Novagen), 1:50 (v/v) protease inhibitor cocktail (Roche). 20 µg lots of protein were fractionated by SDS-PAGE (Novex) and radiolabeled proteins detected by Phosphorimager after drying (Typhoon Molecular Imager FX, GE Healthcare), and quantified using Image J software.

**Cellular Oxygen Consumption**: The Oxygen Consumption rate (OCR) of adherent fibroblasts was assayed with a XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Briefly, $4x10^4$ proliferating fibroblasts were seeded in microplates (Seahorse Bioscience) in 250 µl of prewarmed growth medium (DMEM, GIBCO) and incubated 37°C /5% $CO_2$ for 6 h. Subsequently, the medium was removed and replaced with assay medium and the cells incubated for 30 min in a 37°C non-$CO_2$ incubator. After taking an OCR baseline measurement, 1 µM oligomycin, 1.2 µM carbonylcyanide-4-trifluorometho-xyphenylhydrazone (FCCP) and 1 µM rotenone were added sequentially. For normalization, protein concentration in each well was determined using the Lowry assay (Biorad).

**Generation of a cell line carrying inducible transgenic TWINKLE:** A cDNA of TWINKLE was cloned into vector pcDNA5/FRT/TO (Invitrogen). HEK293T cells (Invitrogen) were transfected with 0.2 µg of pcDNA5.Twinkle and 1 µg of pOG44, using lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Prior to transfection HEK293T cells were cultured in DMEM supplemented with 10% tetracycline-free fetal bovine serum, 15 µg/ml blasticidin and 100 µg/ml zeocin. Transformants were grown in 15 µg/ml blasticidin, 100 µg/ml hygromycin. Transgene expression was induced for 72 h at a dose of 20 ng/mL doxycycline.

## Supplemental Tables

| 5' end | 3' end | mtDNA | Incidence | Length (nt) | polyA |
|---|---|---|---|---|---|
| 15476 | 16192 | ML6-GE | 1 | 716 | |
| 15476 | 16190 | ML6 | 4 | 714 | |
| 15516 | 16189 | ML6 | 1 | 673 | |
| 15561 | 16189 | ML6 | 1 | 628 | |
| 15581 | 16190 | ML6 | 1 | 609 | 11 |
| 15580 | 16189 | ML6 | 1 | 609 | |
| 15584 | 16190 | ML6 | 1 | 606 | |
| 15586 | 16190 | ML6 | 1 | 604 | 13 |
| 15593 | 16190 | ML6 | 1 | 597 | |
| 15595 | 16190 | ML6 | 2 | 595 | 1 |
| 15599 | 16189 | ML6 | 1 | 590 | |
| 15600 | 16190 | ML6 | 1 | 590 | 2 |
| 15601 | 16189 | ML6 | 2 | 588 | |
| 15609 | 16188 | ML2-GE | 1 | 579 | |
| 15660 | 16190 | ML6 | 1 | 530 | 11 |
| 15671 | 16097 | ML6 | 1 | 426 | |
| 15648 | 16091 | ML6 | 1 | 443 | |
| 15620 | 16087 | ML1-GE | 1 | 467 | |
| 15607 | 16084 | ML1-GE | 1 | 477 | |
| 15476 | 16072 | ML1-GE | 1 | 596 | |
| 15659 | 16067 | ML1-GE | 1 | 408 | |
| 15606 | 16046 | ML2-GE | 5 | 440 | |
| 15475 | 16039 | ML2-GE | 1 | 564 | |
| 15745 | 16039 | ML2-GE | 1 | 294 | |
| 15478 | 16031 | ML6 | 1 | 553 | |
| 15628 | 16007 | ML6 | 1 | 379 | 15 |
| 15667 | 15991 | ML1-GE | 3 | 324 | |
| 15667 | 15989 | ML1-GE | 1 | 322 | |
| 15595 | 15988 | ML1-GE | 1 | 393 | |
| 15592 | 15981 | ML1-GE | 1 | 389 | |

**Table S1. Cloned sequences of mouse mitochondrial R-loops (LC-RNA), after circularized RT-PCR.** 5′ and 3′ ends denote the ends of the RNAs according to the revised reference sequence of murine mtDNA (7). Blue shading - RNAs with a 3′ end mapping to LSP; purple shading - 5′ ends at TAS; green shading - 5′ ends near Ori-b (see text for details). RNAs were derived directly from purified preparations (numbered chronologically (numbers not listed were used in other studies)) of mouse liver (ML) mtDNA or from gel-extracted material (GE) from the region where the mitochondrial R-loop is predicted to migrate. PolyA – the number of adenosine residues at the 3′ end of the RNA that do not match the reference sequence and are therefore inferred to be added post-transcriptionally.

| | Family 2, III.9 | Family 2, III.10 | Family 2, III.11 | Family 2, III.8 | Family 1, II.8 | Family 1, II.6 | Family 1, II.1 |
|---|---|---|---|---|---|---|---|
| Status | Affected | Affected | Affected | Affected | Affected | Unaffected | Affected |
| Unique reads | 30293109 | 23468401 | 28223264 | 31470349 | 30329427 | 36841048 | n/a |
| % Aligned | 0.79 | 0.78 | 0.77 | 0.78 | 0.78 | 0.78 | n/a |
| min 10x depth % | 0.79 | 0.70 | 0.75 | 0.79 | 0.77 | 0.82 | n/a |
| Tot variants | 21372 | 20316 | 20930 | 21478 | 21542 | 22161 | 20075 |
| Homozygous variants | 8696 | 8298 | 8741 | 8642 | 8535 | 8795 | 7690 |
| Non-syn variants* | 4308 | 4106 | 4358 | 4340 | 4228 | 4335 | 3542 |
| Novel variants | 47 | 36 | 63 | 61 | 56 | 62 | 17 |

**Table S2. Exome Sequence Data Summary.** Summary of all the variants for each patient (affected and unaffected) where exome sequencing was performed. Results from Exome Sequencing of Families 1 and 2 with variants identified. *includes stop, gain, indel and splice variants. **Total shared variants: 1 RNASEH1 V142I.** All three unaffected family members sequenced had at least one wild-type RNASEH1 allele.

## Supplemental Figures

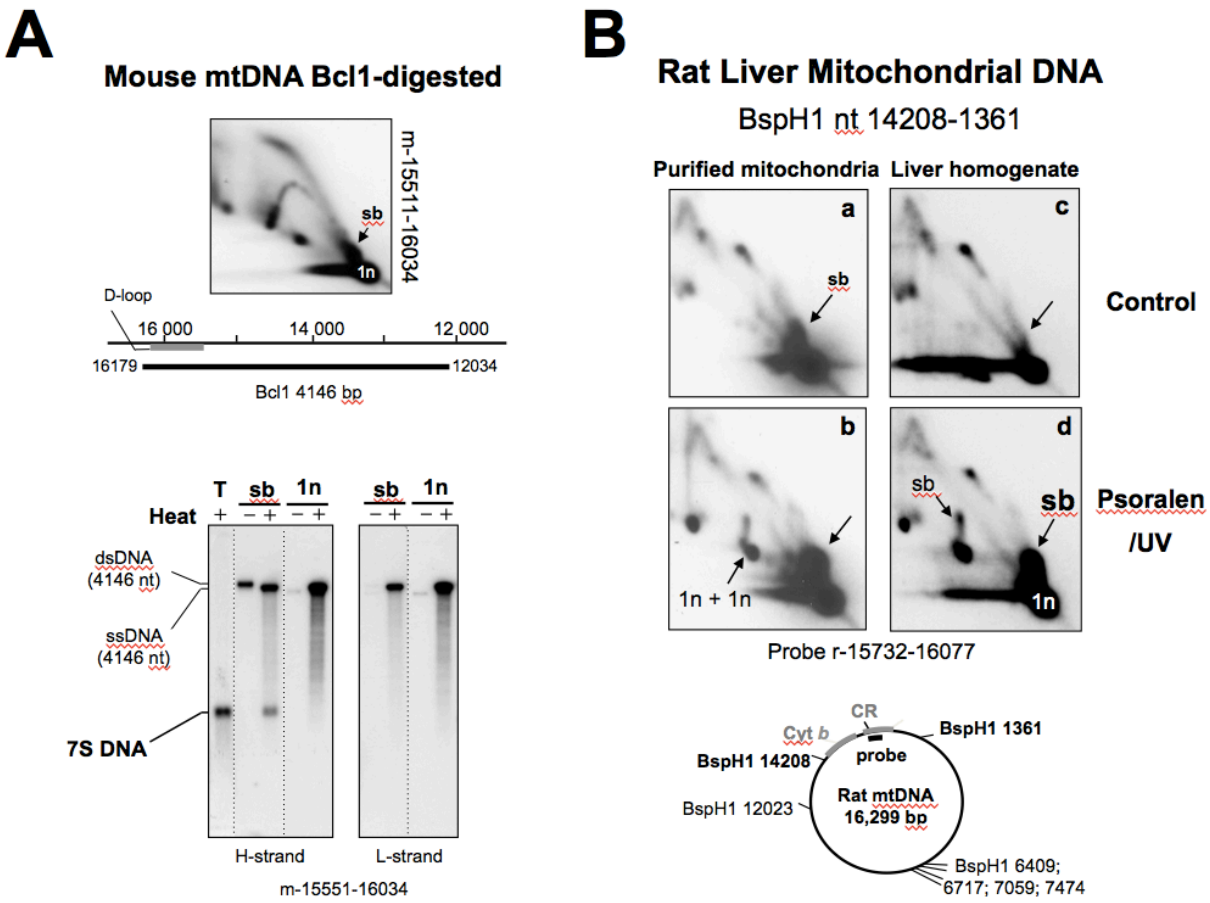


**Figure S1. A**) In contrast to the new preparations of mouse mtDNA (Fig. 1A), no L-strand nucleic acids species (other than the full-length linear restriction fragment, 1n) were detected when small bubble (sb) structures were gel-purified from the base of an initiation arc, denatured and re-fractionated by 1D-AGE – lower gel image. The L- and H- strand riboprobes for mouse (m) mtDNA spanned np 15,511-16,034. T – total undigested mouse mtDNA. Panel A is adapted from (8). **B**) Psoralen-ultraviolet crosslinking markedly increased the signal (i.e. the stability or integrity) of the small bubble-like structures. DNA from crosslinked (b and d) or control (a and c) rat mitochondria or liver homogenate, digested with BspH1, prior to 2D-AGE and hybridization to a probe spanning np 15,732-16,077 of the Control Region (CR) of rat (r) mtDNA. Crosslinking also prevented cleavage at the np 14,208 BspH1 site in a fraction of molecules producing a new spot (1n [14,208-1,361] + 1n [12,023-14,208]) and an accompanying small bubble (sb) arc. Cyt *b* – cytochrome *b* gene. Panel B is adapted from (9). Based on the new findings reported here the small bubbles comprise D-loops and R-loops in similar amounts.

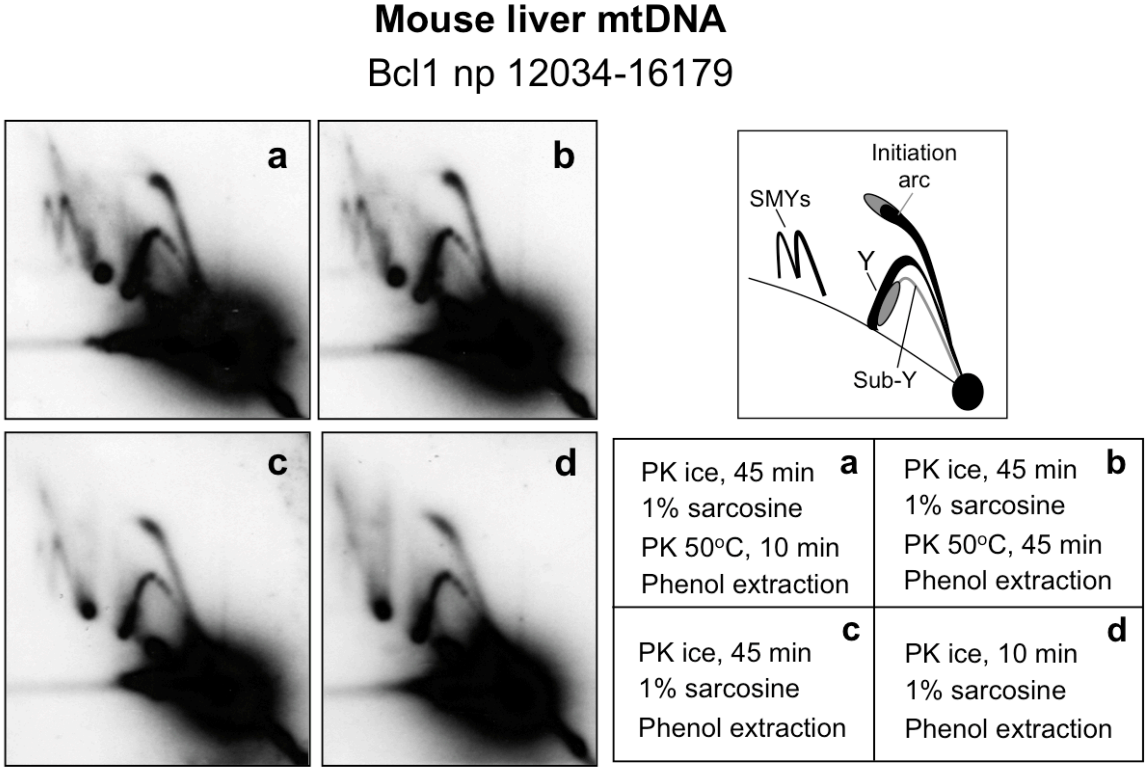


**Figure S2. Purification of mouse liver mitochondrial DNA at 4°C improves the quality of the replication intermediates.** Purified mtDNA from mouse liver mitochondria (see methods) were digested with Bcl1 and blot hybridized to probe np 15,551-16,034, after 2D-AGE. Omission of the proteinase K (PK) step at 50°C after detergent (sarcosine) lysis yielded longer initiation arcs (the final portion indicated in gray in the cartoon), and reduced the signal from the sub-Y arc (again indicated in gray in the cartoon) (panels c and d compared to a and b). 'Retracted' initiation arcs and sub-Y arcs are attributable to the degradation of RNA associated with replicating mtDNA molecules (detailed in (3)). Hence the RNA/DNA hybrids of mtDNA are adjudged to be better preserved using protocols c and d compared to a and b. Therefore, the 50°C step was avoided for the analysis of RNA/DNA hybrids in the control region of murine liver mtDNA in this report. Y – standard replication fork arc; Sub-Y – partially degraded Y arc, SMYs – slow-moving Y arcs attributable to RNA/DNA hybrids at Bcl1 sites (nps 7,084, 11,329 and 12,034) outside the control region (detailed in (3)).

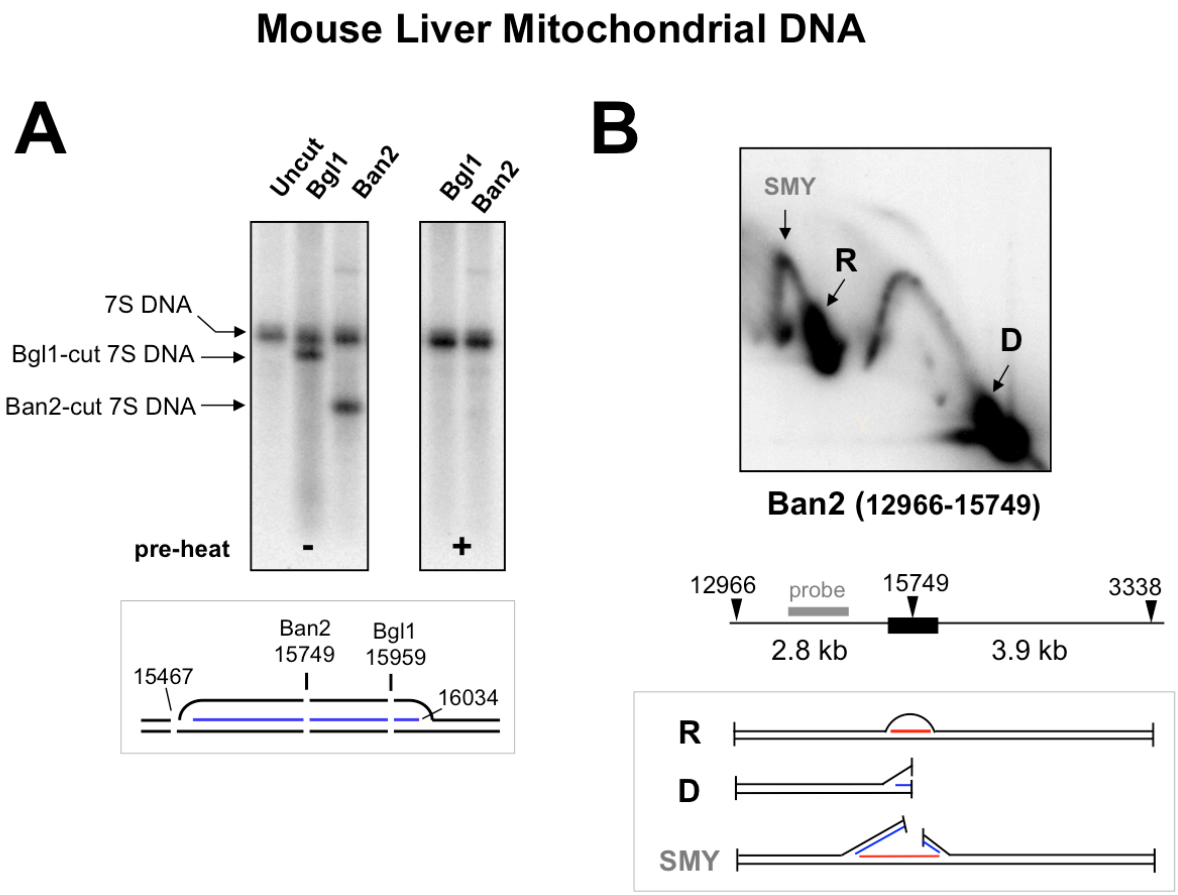


**Figure S3. Restriction enzymes, Bgl1 and Ban2 cleave 7S DNA in the form of a D-loop, but not free 7S DNA; they also cut some but not other small bubble structures associated with fragments of mtDNA containing the control region.** (**A)** Purified murine liver mtDNA was digested with Bgl1 or Ban2, in one case the samples were pre-heated to 70$^{o}$C for 15 min to release 7S DNA before loading (preventing cleavage by the restriction enzymes); all were fractionated by 1D-AGE (1% Tris-acetate) and blot hybridized to a probe within the CR (np 15,551-16,034). (**B)** Murine liver mtDNA digested with Ban2 and blot hybridized to probe np 14,903-15,401, after 2D-AGE. Interpretations are shown in the carton: SMYs arising due to RNA/DNA hybrids formed on replicating mtDNA molecules have been described in detail previously (3); R-loops (R) with RNA (red) across np 15,749 prevent Ban2 cleavage at this position, whereas the 7S DNAs of the D-loop (D) (and nascent strands – blue) are cleaved by Ban2.

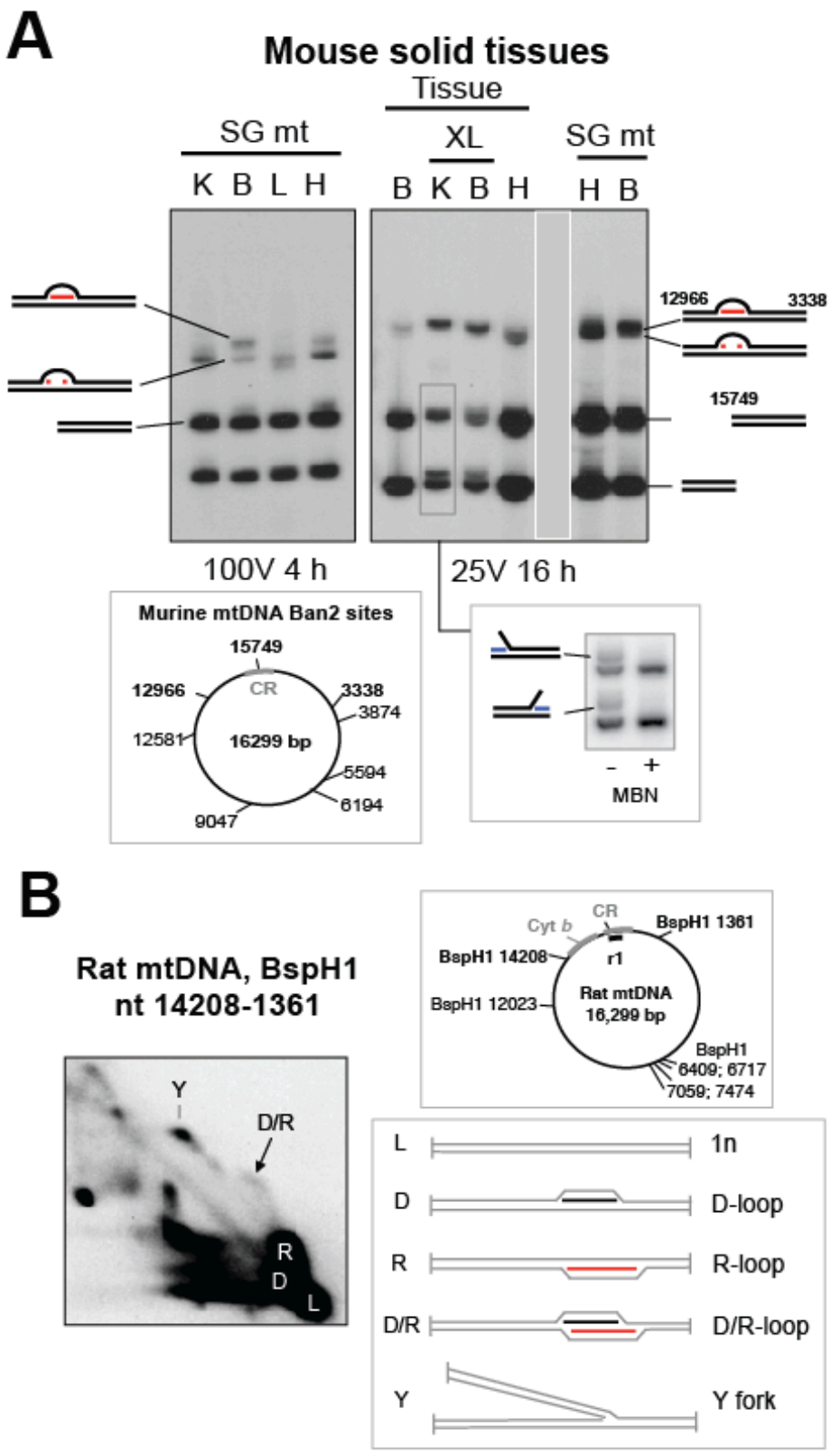


**Figure S4. D-loop and R-loop species in mouse and rat tissues.** (**A**) Mitochondrial DNA molecules refractive to Ban2 digestion extracted from a variety of mouse tissues. Mitochondrial DNA extracted from sucrose gradient purified mitochondria (SG mt) of B - brain, H - heart, K - kidney or L - liver mitochondria was digested with Ban2 and fractionated by 1D-AGE (1% Tris-borate), blot hybridized to a probe spanning np 15,450-16,034 of the mouse mitochondrial genome. Separately, total tissue DNA was extracted

from murine kidney and brain after dounce homogenization and 10 μM, 10 psoralen/UV cross-linking at 365 nm for 10 min (XL), and subjected to the same Ban2 digestion and fractionation. Interpretations are shown to the sides of the gel images. R - R-loops with RNA in red across np 15,749 prevents Ban2 cleavage at this position, whereas the 7S DNAs (blue), of the D-loop (D), are cleaved by Ban2. A gray box covers one lane that does not add materially to the results. The preparation of liver mitochondria was slower than for the other tissues, which may account for the few surviving R-loops on this occasion in this tissue. The species assigned as D-loop containing fragments were, as expected, modified by mung bean nuclease (MBN) treatment that cleaves single stranded DNA (inset). (**B**) Psoralen/UV crosslinking of rat liver mtDNA yields an arc projecting from the R-loop and D-loop spots that is commensurate with synthesis of a dual D/R-loop. DNA from cross-linked rat mitochondria, digested with BspH1, prior to 2D-AGE and hybridization to a probe to the CR (panel B is adapted from (9)).

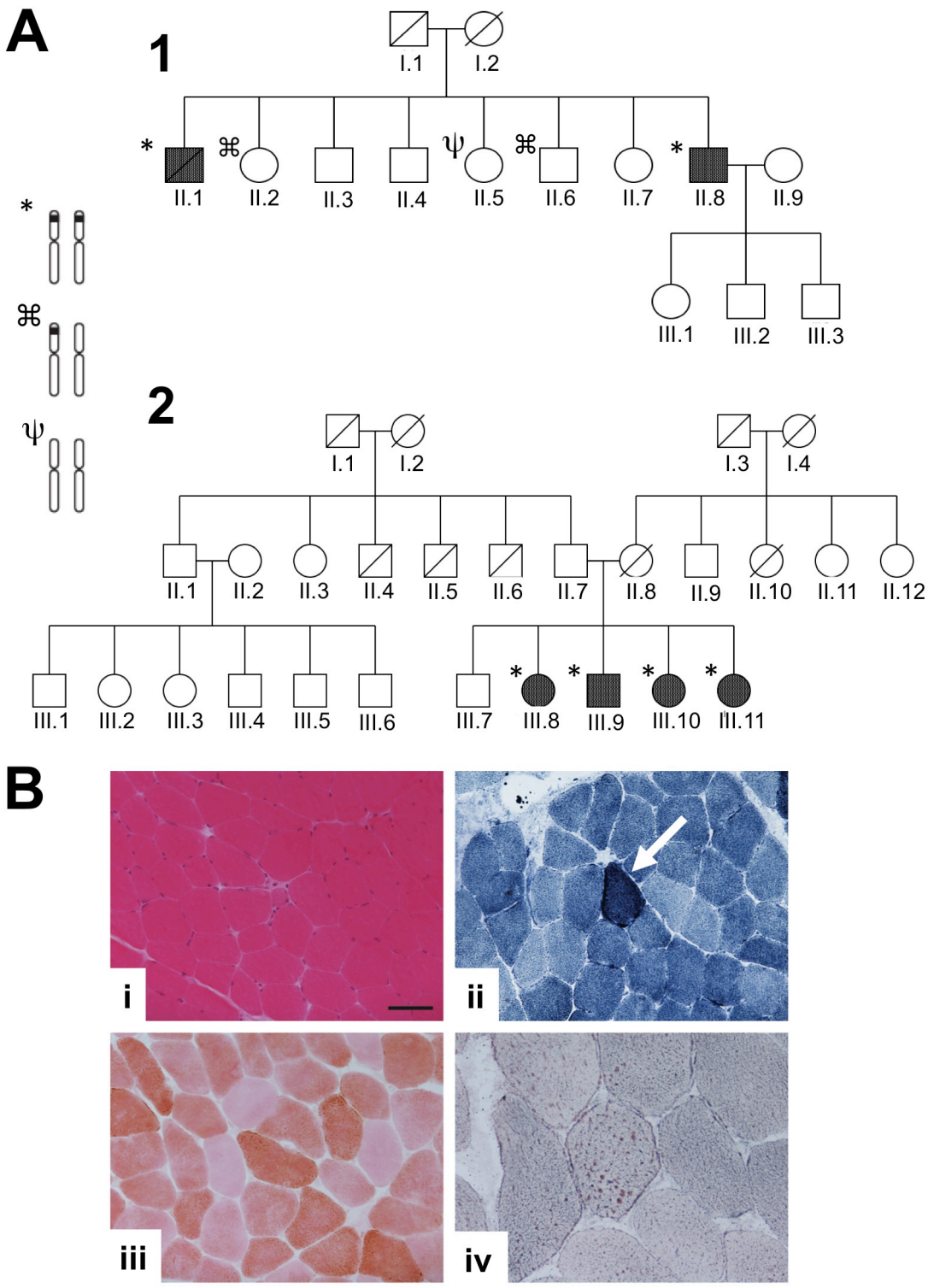


**Figure S5. Pedigrees of the two families (1 and 2) with a homozygous missense mutation in RNASEH1, c.424G>A; p.Val142Ile (A) and muscle histochemistry of an affected patient (II-8) (B).** Chromosome annotations in panel **A**, ψ−homozygous c.424G, ∗−homozygous c.424A (V142I), ⌘−heterozygous individuals. Sparse atrophic fibres visualized in Haematoxylin and Eosin preparation (B-i). Succinate dehydrogenase staining revealed ragged red equivalents (white arrow) (B-ii). Histochemical staining for cytochrome c oxidase demonstrated significant numbers of fibres with deficient enzyme activity (B-iii). Sudan black stain showing increased lipid droplets in central fibers (B-iv). Bar represents 50 μm in B-i-iii and 25 μm in B-iv.

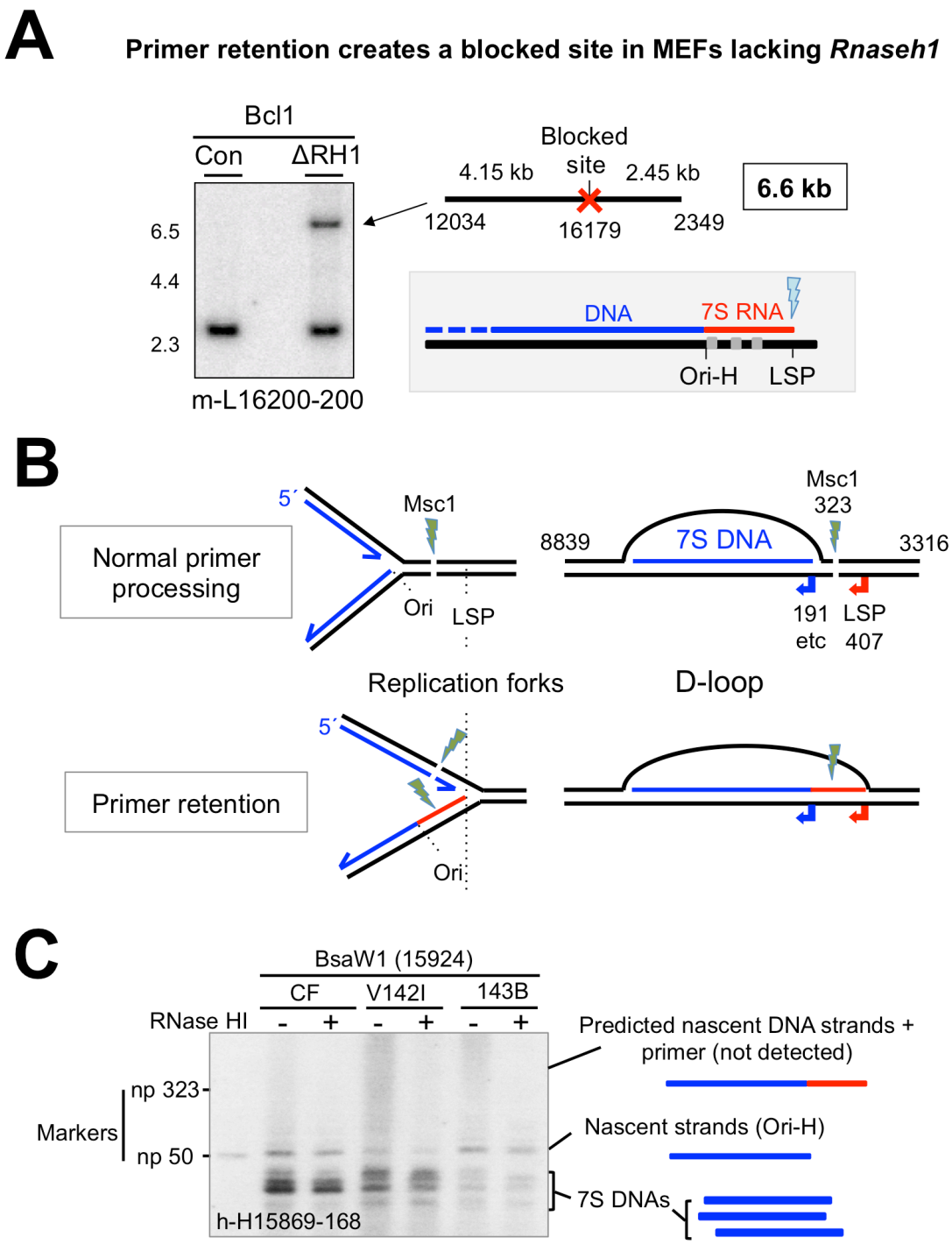


**Figure S6. Primer retention generates RNA/DNA hybrids that are refractive to restriction digestion, but primer retention is not evident in V142I RNase H1 fibroblasts.** (**A**) An inability to remove the RNA spanning LSP to Ori-H (or Ori-b, not illustrated) in MEFs lacking RNase H1 blocks a restriction site at np 16,179, immediately downstream of LSP (adapted from (15)). (**B**) Likewise, primer retention in human mtDNA will result in failure to cut mtDNA molecules at the np 323 Msc1 site. However, np 323 is cut as normal in cells with V142I RNase H1 (Fig. 5E) and nascent strands and 7S DNAs remain the usual length, with and without in vitro RNase HI treatment (Fig. 6B and panel **C** above). (**C**) DNA from V142I and control fibroblasts and 143B cells was BsaW1

digested, denatured, fractionated and probed as per Fig. 6B, additionally some samples were treated (+) with *E. coli* RNase HI.

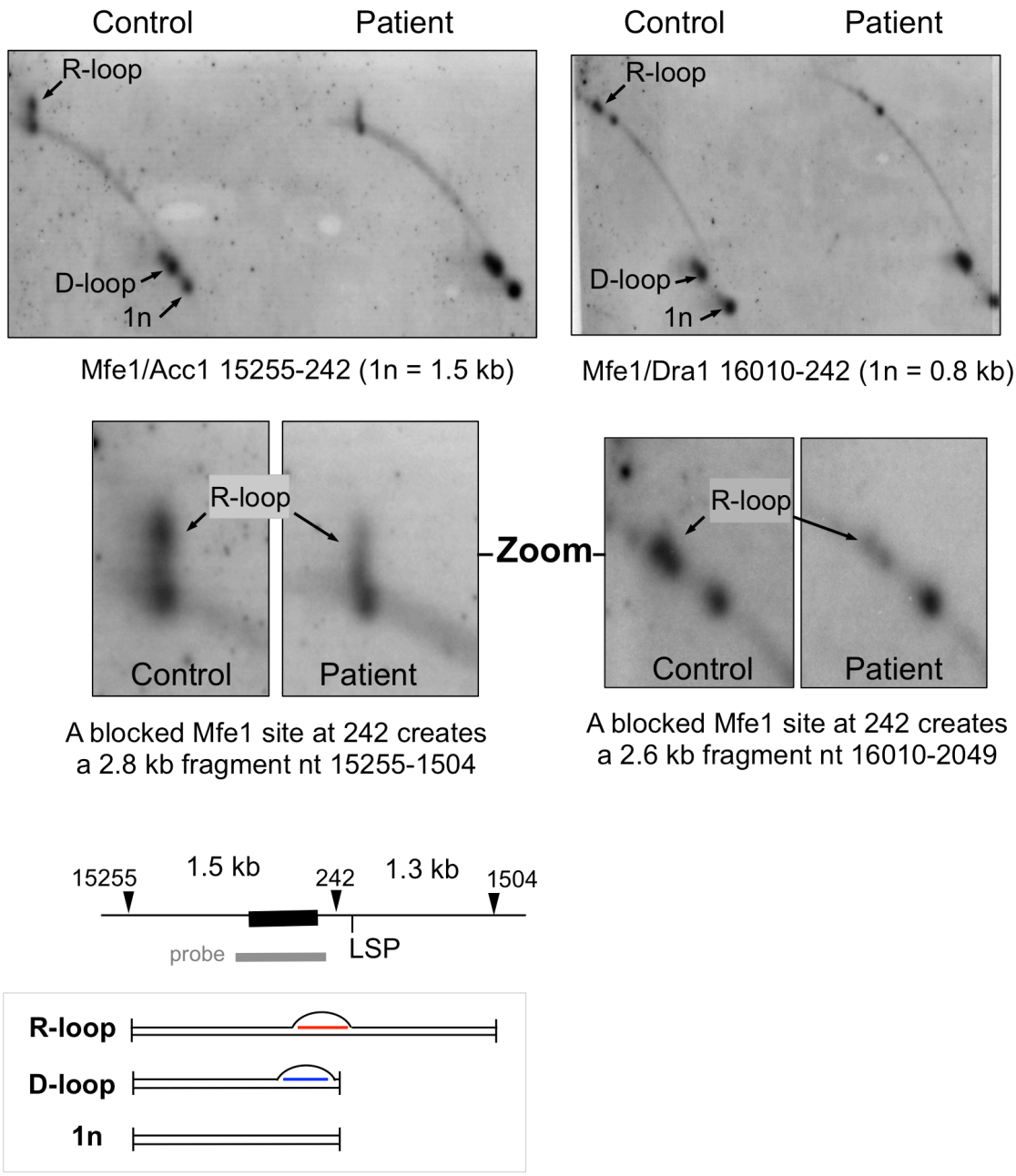


**Figure S7. 2D-AGE analysis indicates R-loops are less abundant in fibroblasts with mutant RNase H1 than controls.** DNAs from human fibroblasts with wild-type (control) or mutant (V142I) RNase H1 were digested with the indicated restriction enzymes, fractionated by neutral 2D-AGE and blot hybridized to riboprobe h-H15869-168. Cells were subjected to UV/psoralen cross-linking (10 μM TMP, 10 min UV 365 nm) prior to nucleic acids isolation. The control fibroblasts for these experiments derived from a different individual to those used in the 1D-AGE, R-loop assay (Fig. 6C). On average R-loop abundance was ~6 fold lower in V142I fibroblasts than the two control cell lines tested (n = 3 independent experiments).

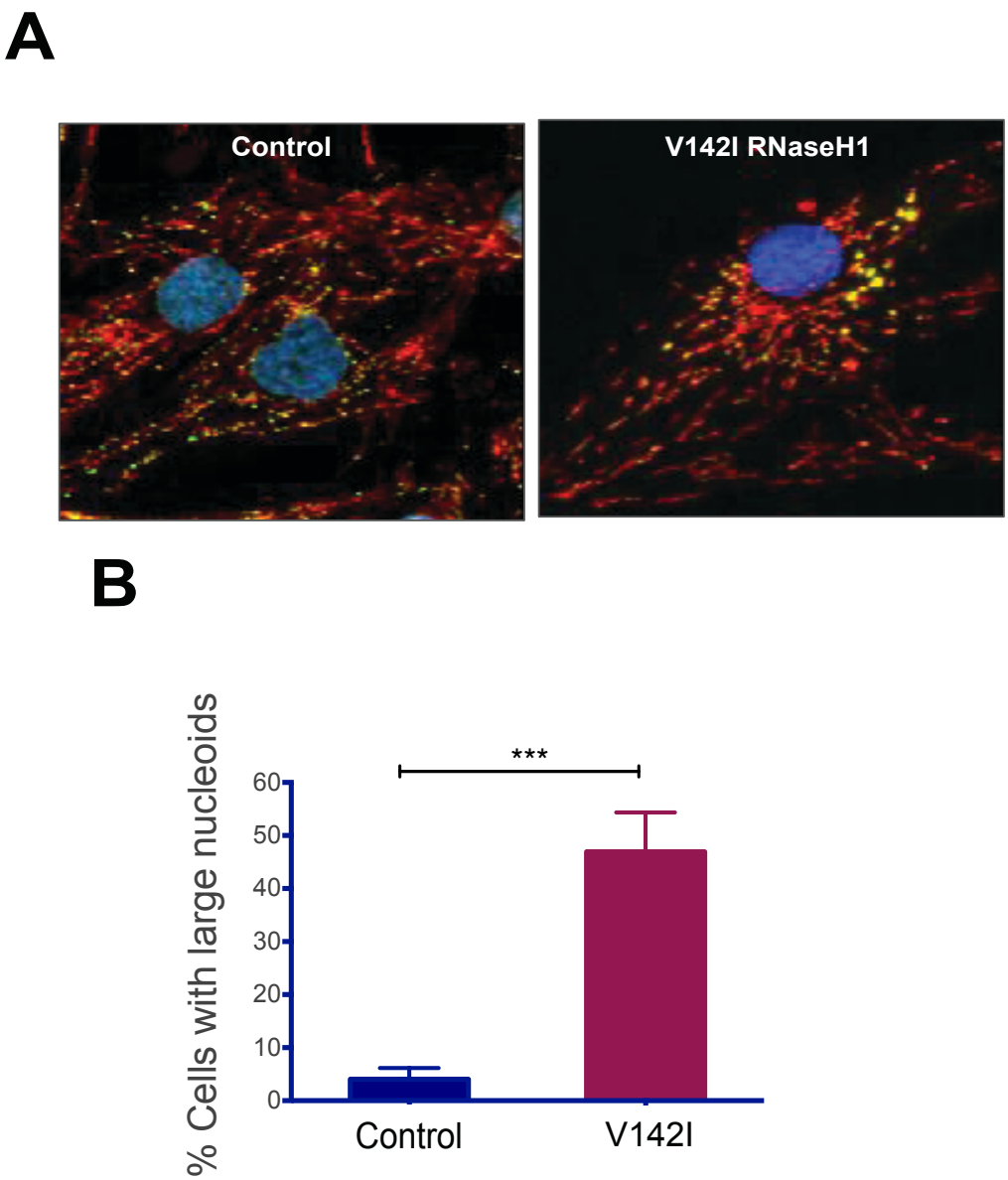


**Figure S8. V142I RNase H1 is associated with enlarged mtDNA foci in human fibroblasts.** (**A**) An example of the pronounced mtDNA clustering in V142I RNase H1 cells compared with control fibroblasts (7301) imaged by confocal microscopy after incubation with antibodies to the mitochondrial outer membrane protein TOM20 (red) and to DNA (green), merged images are shown. The control fibroblasts for these experiments derived from a different individual to those shown in Fig. 7. The number of cells displaying clustering of mtDNA and its extent were much higher in V142I cells compared to six other fibroblast lines (three of which have a potential or confirmed mitochondrial disorder - thereby excluding generalized mitochondrial dysfunction as a cause of mtDNA aggregation). (**B**) The proportion of cells with mtDNA foci larger than the modal 0.3 μm in a representative control cell line compared with fibroblasts with V142I RNase H1 (n = 4 independent experiments, error bars are standard deviation from the mean). Unpaired two-tailed t test with Welch's correction, p = 0.0008.

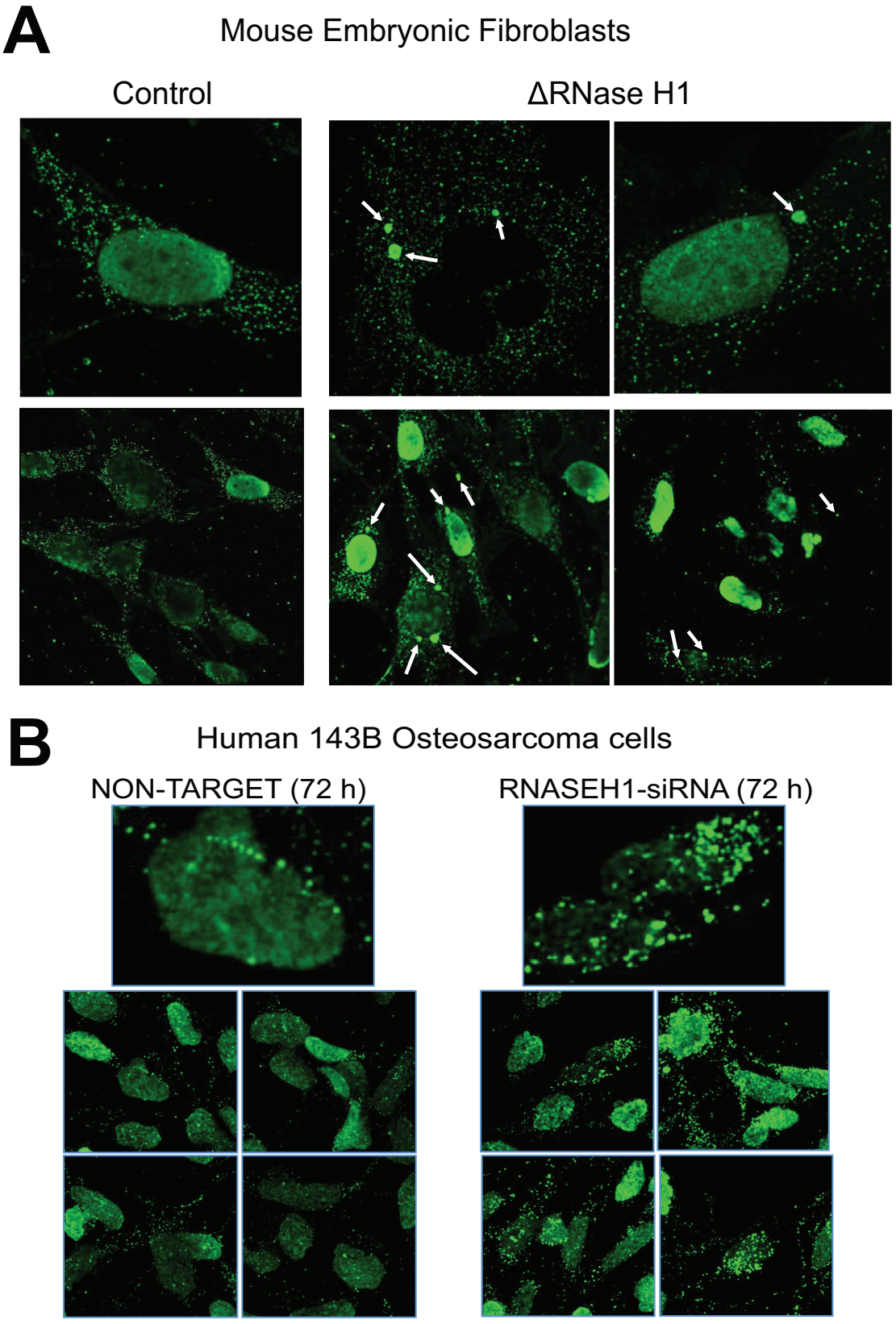


**Figure S9. RNase H1 ablation in murine fibroblasts or knockdown in human osteosarcoma cells results in the formation of enlarged mitochondrial DNA foci.** MEFs were cultured without (Control) or for 5 or 7 days with 4-hydroxy-tamoxifen (ΔRNase H1) (as previously (10)). Human 143B cells were transfected with a non-target siRNA or siRNA to RNASEH1, and imaged 72 hours later.

Immunocyto-chemistry was performed with anti-DNA antibodies (green). White arrows indicate examples of enlarged mtDNA foci.

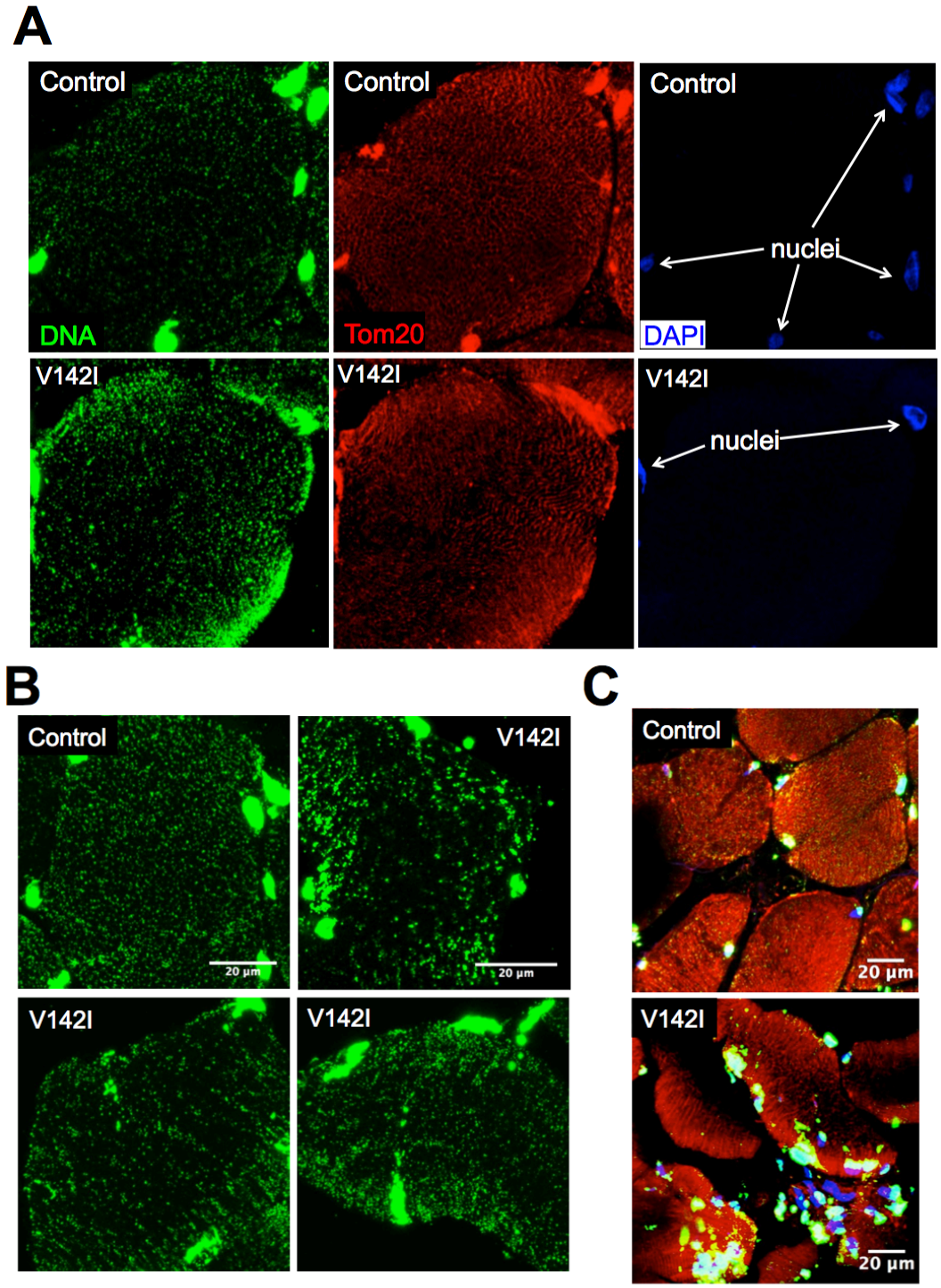


**Figure S10. V142I RNase H1 quiescent cells and muscle display mtDNA disorganization and aggregation.** (**A**) Individual components of the merged images of Fig. 8B and DAPI-labeled nuclei. (**B**) additional images of individual muscle fibers labeled with anti-DNA antibodies (green); and (**C**) muscle sections of a control and the index case with V142I RNase H1. Anti-DNA - green, Anti-TOM20 (mitochondria) - red. The sizes of the mtDNA foci in muscle sections of the control were 0.25-0.38 μm, similar to those of control fibroblasts (Fig. 7). The nuclei in muscle are denser than in fibroblasts and other cultured cells, which we infer accounts for the non-specific labeling of some nuclei with anti-TOM20. Purple signal in (**C**) is autofluorescence.

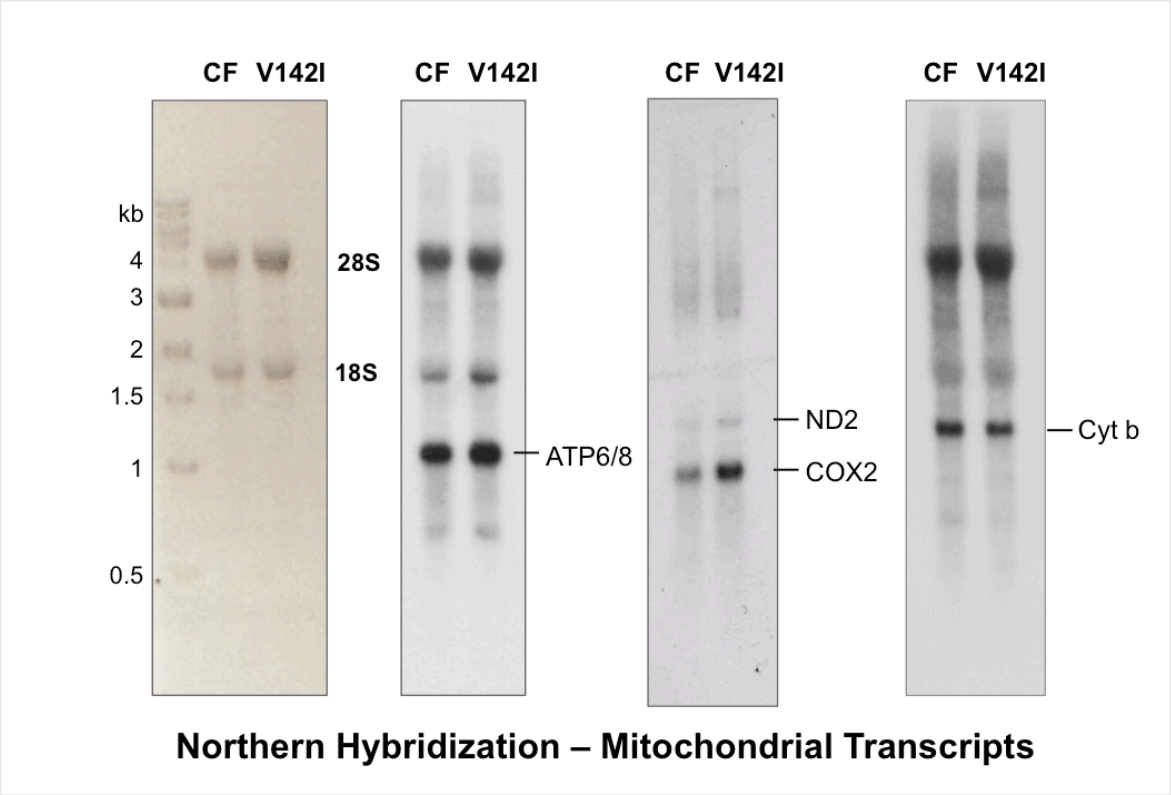


**Figure S11. V142I RNase H1 does not decrease the levels of four mature mitochondrial mRNAs or their precursors.** RNA was isolated from patient-derived (V142I) and control (CF – L1068) fibroblasts. Five microgram lots were fractionated by 1D-AGE in MOPS buffer (see Materials and Methods) and blot hybridized to ribobprobes complementary to mitochondrial mRNAs encoding ATP synthase subunits 6 and 8 (ATP6/8), NADH dehydrogenase 2 (ND2), cytochrome oxidase subunit 2 (COX2) and cytochrome b (cyt b). The ATP6/8 and cyt b probes also bound non-specifically to the abundant 28S and 18S rRNAs of cytosolic ribosomes.

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