Supplementary Materials for

Mammalian RNase H1 directs RNA primer formation for mtDNA replication initiation and is also necessary for mtDNA replication completion

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Fig. S1. Tissue-specific \textit{Rnaseh1} knockout mice. (A), Schematic representation of \textit{Rnaseh1} cDNA. RT-PCR analysis of \textit{Rnaseh1} transcripts in heart of control (L/L) and knockout (L/L, cre) mice. (B), \textit{Rnaseh1} transcript levels in heart of 8- and 24-week old L/L and L/L, cre mice by RT-qPCR. Actin B was used as a housekeeping gene control. Data are represented as mean ± SEM; Welch’s t-test; n=5 animals per group; *p < 0.05; **p < 0.01; ***p < 0.001. (C), Survival curve of L/L (n=15) and L/L, cre mice (n=15). Log-rank (Mantel-Cox) test was performed; p<0.0001. (D), Southern blot analysis of SacI-digested total DNA from skeletal muscle of 16-week-old L/L and L/L, cre mice. Plasmid pAM1, containing whole mtDNA sequence was used as a probe. 18S rDNA was used as a loading control.
Fig. S2. Linear deleted mtDNA in *Rnaseh1* knockout hearts. (A), Schematic representation of mouse mtDNA with SacI, XhoI, BglII and EagI restriction sites. O_H indicates the heavy-strand origin of replication and O_L stands for the light-strand origin of replication. Probes used for following Southern blot analyses are represented in blue (pAM1) and in red (12S rDNA). Linear deleted mtDNA molecule spans the region highlighted in green. Southern blot analyses of heart mtDNA from control (L/L) and knockout (L/L, cre) 8-week-old mice and *Mgme1* ^−/−^ (knockout) and *Mgme1* ^+/−^ (control) mice after digestion with SacI (B) or XhoI (C). Red arrows represent the DNA migration pattern of linear deleted mtDNA fragments in *Rnaseh1* and *Mgme1* knockout hearts. Southern blot analyses of L/L and L/L, cre heart mtDNA from 8-week-old mice. Samples were digested with one of the following enzymes: XhoI, SacI, BglII or EagI. Membrane was first hybridized with 12S rDNA probe (D), stripped and hybridized with pAM1 probe (E and F). Red arrows represent the DNA migration pattern of linear deleted mtDNA in *Rnaseh1* knockout hearts.
Fig. S3. OXPHOS dysfunction in Rnaseh1 knockout mice. (A), Steady-state protein levels in mitochondria isolated from heart of 8-, 16- and 24-week-old control (L/L) and knockout (L/L, cre) mice by western blot analysis. (B), COX/SDH staining of skeletal muscle tissue from 8- and 24-
week-old \(L/L\) and \(L/L, cre\) mice. Scale bar 50 \(\mu\)m. (C), In gel activity of complex I, II and IV in mitochondria isolated from heart of 8- and 24-week-old \(L/L\) and \(L/L, cre\) mice.

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**Fig. S4. mtDNA assessment in patient skeletal-muscle biopsy specimens.** Quantification of mtDNA deletions (A) and mtDNA copy number (B) in skeletal muscle biopsies of healthy individuals (Controls) and Y29C (RNASEH1) and S188YfsX6 (MGME1) patients. Data are shown as scatter plot with mean (± SD). Amount of mtDNA deletions is expressed as percentage of ND4 (major arc, commonly deleted) on ND1 (minor arc, commonly not deleted). One-way ANOVA with Dunnett’s multiple comparisons test, \(p<0.05\). (C), COX/SDH staining of skeletal muscle from the S188YfsX6 (MGME1) patient. A few COX-negative fibers are seen as indicated by black arrows. Magnification 20x, scale bar 50 \(\mu\)m. (D), Quantification of 7S DNA in skeletal muscle biopsies of healthy individuals (Controls) and Y29C (RNASEH1) and S188YfsX6 (MGME1)
patients. Data are shown as scatter plot with mean (± SD). One-way ANOVA with Dunnett’s multiple comparisons test, p<0.05.

Table S1. Key reagents and resources used in this study.

| Reagent                                           | Source                      | Identifier     |
|---------------------------------------------------|-----------------------------|----------------|
| Amersham ECL Western Blotting Detection Reagent   | GE Healthcare               | Cat# RPN2106   |
| anti-AIF                                          | Abcam                       | Cat# ab16501   |
| anti-GAPDH                                        | Abcam                       | Cat# ab8245    |
| anti-Histone 3                                    | Sigma Aldrich               | Cat# H0164     |
| anti-HSP60                                        | Enzo Life                   | Cat# ADI-SPA-807-E |
| anti-LRPPRC                                       | Sigma Aldrich               | Cat# HPA 036409 |
| anti-mouse IgG horseradish peroxidase             | GE Healthcare               | Cat# NA9310V   |
| anti-OXPHOS human                                 | Abcam                       | Cat# ab110411  |
| anti-OXPHOS rodent                                | Abcam                       | Cat# ab110413  |
| anti-rabbit IgG horseradish peroxidase            | GE Healthcare               | Cat# NA9340V   |
| anti-RNase H1                                     | Proteintech                 | Cat# 15606-1-AP|
| anti-TFAM                                         | Abcam                       | Cat# ab131607  |
| anti-TIM 22                                       | Proteintech                 | Cat# 14927-1-AP|
| anti-TOM 20                                       | Cell Signalling             | Cat# 13929S    |
| anti-VDAC                                         | Abcam                       | Cat# ab14734   |
| BamHI                                             | New England Biolabs         | Cat# R0136S    |
| BclI                                              | New England Biolabs         | Cat# R0160S    |
| Digitonin                                         | Merck                       | Cat# 300410    |
| DMEM GlutaMAX                                     | Thermo Fisher Scientific    | Cat# 31966021  |
| EcoRI                                             | New England Biolabs         | Cat# R0101S    |
| Gibco Fetal Bovine Serum                          | Thermo Fisher Scientific    | Cat# 10500064  |
| Gibco Penicillin/Streptomycin                     | Thermo Fisher Scientific    | Cat# 15-140-122|
| Iodonitrotetrazolium chloride                     | Merck                       | Cat# 110406    |
| PerfectHyb Plus Hybridization buffer              | Merck                       | Cat# H7033     |
| Purified anti-POLRMT                              | N.-G.Larsson                | Kuhl et al, 2014 |
| Item                                             | Supplier               | Catalog Number |
|--------------------------------------------------|------------------------|----------------|
| Rapid hyb Buffer                                 | Cytiva                 | Cat# RPN1635   |
| Sacl-HF                                         | New England Biolabs    | Cat# R3156S    |
| Xhol                                             | New England Biolabs    | R0146S        |
| Eacl-HF                                         | New England Biolabs    | R3505S        |
| BclII                                           | New England Biolabs    | R0144S        |
| Triton X-100                                     | Merck                  | Cat# T8787    |
| Trizol reagent                                   | Thermo Fisher Scientific | 15596026     |
| T4 Polynucleotide Kinase                         | New England Biolabs    | Cat# M0201    |
| RNase H                                         | New England Biolabs    | Cat# M0297S   |
| Human PolG A&B                                   | Posse et al, 2019      | N/A           |
| Human RNase H1 and mutants                        | Al-Behadili et al, 2018, this work | N/A |
| Human POLRMT                                     | Posse et al, 2019      | N/A           |
| Human TFB2M                                      | Posse et al, 2019      | N/A           |
| Human mtSSB                                      | Posse et al, 2019      | N/A           |
| Human TFAM                                       | Posse et al, 2019      | N/A           |

**Critical Commercial Assays**

| Item                                             | Supplier               | Catalog Number |
|--------------------------------------------------|------------------------|----------------|
| Cell Fractionation Kit                            | Abcam                  | Cat# ab109719  |
| Gentra Puregene Tissue Kit                        | Qiagen                 | Cat# 158667)   |
| High-Capacity cDNA Reverse Transcription Kit      | Thermo Fisher Scientific | 4368813        |
| miRNeasy Mini Kit                                 | Qiagen                 | Cat# 217004    |
| Prime-It II Random Primer Labeling Kit            | Agilent                | Cat# 300385    |
| TaqMan™ Universal Master Mix II, with UNG         | Thermo Fisher Scientific | 4440038        |
| TURBO DNA-free™ Kit                               | Thermo Fisher Scientific | AM1907         |

**Experimental Models: Organisms/Strains**

| Item                                             | Supplier               | Catalog Number |
|--------------------------------------------------|------------------------|----------------|
| Mouse: C57BL/6N                                   | Charles River          | N/A            |
| Mouse: Ckmm Cre                                   | The Jackson Laboratory  | N/A            |
| Mouse: Rnaseh1<sup>+/−</sup>                       | This work              | N/A            |
| Mouse: Rnaseh1<sup>loxP/loxP</sup>                 | TACONIC                | Custom order   |
| Mouse: Mgme1<sup>−/−</sup>                         | N.-G. Larsson          | Matic et al, 2018 |
| Mouse: Tefm<sup>loxP/loxP, +Ckmm-cre</sup>        | N.-G. Larsson          | Jiang et al, 2019 |
| Primers                                                                 | Supplier         | Description                              |
|------------------------------------------------------------------------|------------------|------------------------------------------|
| CACGACCAAGTGACAGCAAT                                                   | Sigma-Aldrich    | Cre-FW (genotyping)                      |
| AGAGACGGAAATCCCATCGCT                                                  | Sigma-Aldrich    | Cre-RV (genotyping)                      |
| CCTAGGATTGGAGTTAAGGC                                                   | Sigma-Aldrich    | Rnaseh1 primer 29 FW (genotyping)        |
| TCTAATGTCCCTTAGAGTAGGCTTC                                              | Sigma-Aldrich    | Rnaseh1 primer 30 RV (genotyping)        |
| GCAACCTGCACATAGATTCG                                                  | Sigma-Aldrich    | Rnaseh1 primer 25 FW (genotyping)        |
| TTGCGGGCTCGGGATGTTC                                                  | Sigma-Aldrich    | Rnaseh1 exon 1 FW (RT PCR)               |
| CTTTGCTCACTACAGCCGAG                                                  | Sigma-Aldrich    | Rnaseh1 exon 3 RV (RT PCR)               |
| GACATATAATATTTAACTATCA                                                | Sigma-Aldrich    | 7S RNA primer (northern blot)            |
| AACCTATGTGCTGATCAACC                                                  | Sigma-Aldrich    | O1 FW (Southern blot probe 2DNAGE)       |
| ATCAATGGGTCCAGTCATAA                                                | Sigma-Aldrich    | O1 RV (Southern blot probe 2DNAGE)       |
| GTAATCATCAAC                                                          | Sigma-Aldrich    | O2 FW (Southern blot probe 2DNAGE)       |
| GCCCTAGGTGATTGGGTTTTC                                                 | Sigma-Aldrich    | O2 RV (Southern blot probe 2DNAGE)       |
| TGACTTGTCCTCCACTAATAATCGGAG                                             | Sigma-Aldrich    |                                           |
| CCCAAAGAATCAGAACAGATGCTG                                               | Sigma-Aldrich    |                                           |
| ARC140: /5AmMC6/ACACTCTTTCC                                          |                  | HydEn seq primer                         |
| CTACACGACGCTCTCTCCGATCT                                                |                  |                                           |
| ARC76: GTGACTGGAGTTCGAC                                                |                  | HydEn seq primer                         |
| GTGTGCTCTCTCCGATCTCTNNNN*N*N                                          |                  |                                           |
| ARC77: AGATCGGAAAGAGCAC                                              |                  | HydEn seq primer                         |
| GTCTGAACTCCAGTCAACC                                                    |                  |                                           |
| ARC78CAAGCAGAAGACCGCATA                                               |                  | HydEn seq primer                         |
| GAGATCGTGTATGTGACTGGAGTTC                                              |                  |                                           |
| AGACGTGTGTCTCTCCGATCT                                                  |                  |                                           |
| ARC84 CAAGCAGAAGACCGCATA                                               |                  | HydEn seq primer                         |
| CGAGATACATCCGTTGACTGGAGTTC                                             |                  |                                           |
| CAGACGTGTGTCTCTCCGATCT                                                  |                  |                                           |
| ARC85 CAAGCAGAAGACCGCATA                                               |                  | HydEn seq primer                         |
| GAGATGCCCTAAGTGTGACTGGAGTTC                                             |                  |                                           |
| AGACGTGTGTCTCTCCGATCT                                                  |                  |                                           |
| ARC86 CAAGCAGAAGACCGCATA                                               |                  | HydEn seq primer                         |
| AGATTTGCTGACTGGAGTTCAG                                                |                  |                                           |
| ACGTGTGCTCTCCGATCT                                                    |                  |                                           |
| ARC87 CAAGCAGAAGACCGCATA                                               |                  | HydEn seq primer                         |
| GAGATCACTGTGACTGGAGTTCAG                                              |                  |                                           |
| GACGTGTGCTCTCCGATCT                                                    |                  |                                           |
| ARC88 CAAGCAGAAGACGGCATACG AGATATTGGCGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
|-----------------|-----------------|
| ARC89 CAAGCAGAAGACGGCATACG AGATGATCTGGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
| ARC90 CAAGCAGAAGACGGCATACG AGATTCAAGTGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
| ARC91 CAAGCAGAAGACGGCATACG AGATCTGATCGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
| ARC93 CAAGCAGAAGACGGCATACG AGATAAGCTAGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
| ARC94 CAAGCAGAAGACGGCATACG AGATGTAAGCTTAGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
| ARC95 CAAGCAGAAGACGGCATACG AGATTACAAGGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT | HydEn seq primer |
| ARC160 AATGATACGGCGACCACCG AGATCTACACCTCGCCTACACTCTT CCCTACACGACGCTCTTCCGATC | HydEn seq primer |

### Taqman Assays and Oligonucleotides

| Assay     | Manufacturer                 | Accession     |
|-----------|------------------------------|---------------|
| m-12S     | Thermo Fisher Scientific     | Mm04260177_s1 |
| m-16S     | Thermo Fisher Scientific     | Mm04260181_s1 |
| m-18S     | Thermo Fisher Scientific     | Mm03928990_g1 |
| m-Actin   | Thermo Fisher Scientific     | Mm01205647_g1 |
| m-Atp6    | Thermo Fisher Scientific     | Mm03649417_g1 |
| m-Atp8    | Thermo Fisher Scientific     | Mm04225236_g1 |
| m-Cox1    | Thermo Fisher Scientific     | Mm04225243_g1 |
| m-Cox2    | Thermo Fisher Scientific     | Mm03294838_g1 |
| m-Cox3    | Thermo Fisher Scientific     | Mm04225261_g1 |
| m-Cyb     | Thermo Fisher Scientific     | Mm04225271_g1 |
| m-Nd1     | Thermo Fisher Scientific     | Mm04225274_g1 |
| m-Nd2     | Thermo Fisher Scientific     | Mm04225288_s1 |
| m-Nd3     | Thermo Fisher Scientific     | Mm04225292_g1 |
| m-Nd4l/4                   | Thermo Fisher Scientific | Mm04225294_s1 |
|---------------------------|-------------------------|---------------|
| m-Nd5                     | Thermo Fisher Scientific | Custom Made_AIHSNT9 |
| m-Nd6                     | Thermo Fisher Scientific | Custom Made_AIVI3E8 |
| m-Rnaseh1                 | Thermo Fisher Scientific | Mm00488036_m1 |

**Recombinant DNA and Oligonucleotides for in vitro assays**

| DNA/Template                                                   | Supplier | Notes                                      |
|---------------------------------------------------------------|----------|--------------------------------------------|
| GCAAATTCGAAGAAGCAGCTTCAAACCT                                 | Eurofins | 80 mer template for RNA:DNA hybrids       |
| GCCGGGGGCTTCTCCCGCCTTTTTTCCTAA                              |          |                                           |
| TAATAAAGAGGACAGATTGAAGC                                       |          |                                           |
| LSP template (nt 1-477 of mtDNA):                             | Posse et al, 2019 | N/A                                       |
| pUC18 DNA with mtDNA seq ligated between BamHI and HindIII    |          |                                           |
| AAAAAAGGCGGGAGGAGCAGGGGGG                                     | Eurofins | 26DNA:26 RNA for RNA:DNA hybrids          |
| GCAGGTTCAGACTTGCTTCTTCGAATTTG                                 |          |                                           |

**Probes**

| DNA/Probe                                                      | Supplier | Notes                                      |
|---------------------------------------------------------------|----------|--------------------------------------------|
| pAM1 /mouse mtDNA cloned in pAcyc177                          | Gift from D.Clayton | N/A                                       |
| mouse 7S DNA for Southern Blot                                 | Matic et al, 2018 | N/A                                       |
| TCCCCAGATTTTCACGAGCAGTTTTTAAC                                  | N/A      | mouse 18S probe for Southern blot          |
| TGCAAGCAACTTTTAATATACGCTATTGGAG                                |          |                                            |
| CTGGAGTACCCGCGCTGTCAGCACGGCCAGGAGA                            |          |                                            |
| CTTGCCCCCTCAAATGGATCCTCGTTTAAGGG                              |          |                                            |
| ATTTAAAGTGGACTCTATTCAATTCAGAGG                                  |          |                                            |
| GCCCTGAAGAGGTCTGTATTTGTATTTT                                  |          |                                            |
| CGTCACACTCCTCCCGGTTGTCGGAGTTGG                                 |          |                                            |
| TAAATTTGCCGCGCCTGCGCTGCTGCTTGGAT                              |          |                                            |
| GTGGTAGCCGCTTTTCTCAGGCTCCTCTTCCCG                              |          |                                            |
| GAATCGAGCTCAGTTATCTAGAGTCACC                                   |          |                                            |
| GTTCACCAGCTATGGACGGCAGCCGGACTACC                                |          |                                            |
| ATCGAAAGGTGTAGGAGGAGACGTTGCA                                   |          |                                            |
| ATGAGGTCGTCGCGCCACGGGGGAGGGGAGGGT                            |          |                                            |
| CGATCGGCAGCCGAGGTATCTAGAGTCACC                                 |          |                                            |
| AAGCCGCCGCCGCCGCACGCCCCGCCGCCGCC                              |          |                                            |
| GAGCCGCCGGAGAGGAGGCTCAAGCTAGGTGT                             |          |                                            |
| TTGATCTGATAAATGCGACGCATCCCCCCCCCC                             |          |                                            |
| GGGAAAGGGGTGCTAGCCGCCGTCGCGCATG                              |          |                                            |
| TATTAGCTCTAGAATCCACAGTTATCCA                                  |          |                                            |
| AGTAGGAGAGGGAGCGGCCAGCACAAAGGA                               |          |                                            |
| ACCATAACGTATTATAATGAGGCCATTCGCA                               |          |                                            |
| GTCACGCTGTACCGGAGGCC                                        |          |                                            |

| mouse 12S probe for Southern blot                             | Jiang et al, 2019 | N/A                                       |
|                         | Author(s)       | Year | Sequence                                                                 |
|-------------------------|-----------------|------|--------------------------------------------------------------------------|
| mouse 5.8S probe for Northern blot | Jiang et al, 2019 | N/A  | N/A                                                                      |
| human 18S probe for Southern blot | Nicholls et al, 2014 | N/A  | N/A                                                                      |
| TTACAGTCAAATCCCTTCTCGTCCCCAT  | N/A             | human 7S DNA for Southern blot | N/A  |
| GGATGACCCCCCTCAGATAGGGGTCCC  |                 |      | N/A                                                                      |
| TTGACCACCATCCTCCGTGAAATCAATA |                 |      | N/A                                                                      |
| TCCCGCACAAGAGTGCTACTCTCTCTGCT |                 |      | N/A                                                                      |
| TCCGGGCCCATAACACTTGGGGGTAGCT  |                 |      | N/A                                                                      |
| AAGTGAACTGTATCCGACATCTGGTTC  |                 |      | N/A                                                                      |
| CTAATTCAGGTCATAAGCCTAAATAG    |                 |      | N/A                                                                      |
| CCCACAGGTTCCTTAAATAAGACATCA   |                 |      | N/A                                                                      |
| ACGATG                         |                 |      | N/A                                                                      |