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Supplemental Information

Dinucleotide Degradation by REXO2 Maintains Promoter Specificity in Mammalian Mitochondria

Thomas J. Nicholls, Henrik Spåhr, Shan Jiang, Stefan J. Siira, Camilla Koolmeister, Sushma Sharma, Johanna H.K. Kauppila, Min Jiang, Volkhard Kaever, Oliver Rackham, Andrei Chabes, Maria Falkenberg, Aleksandra Filipovska, Nils-Göran Larsson, and Claes M. Gustafsson

Figure S1, related to Figure 1.

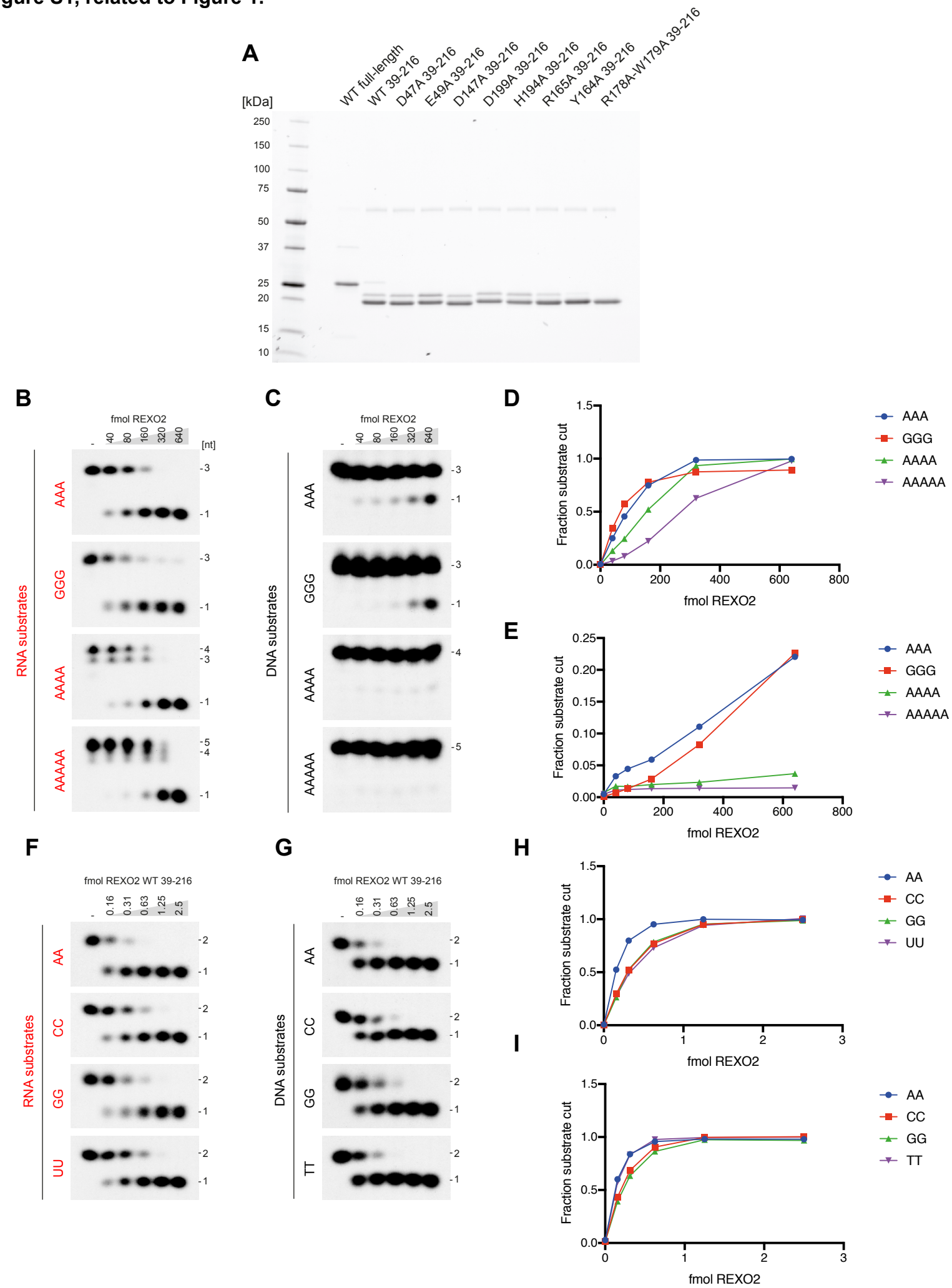
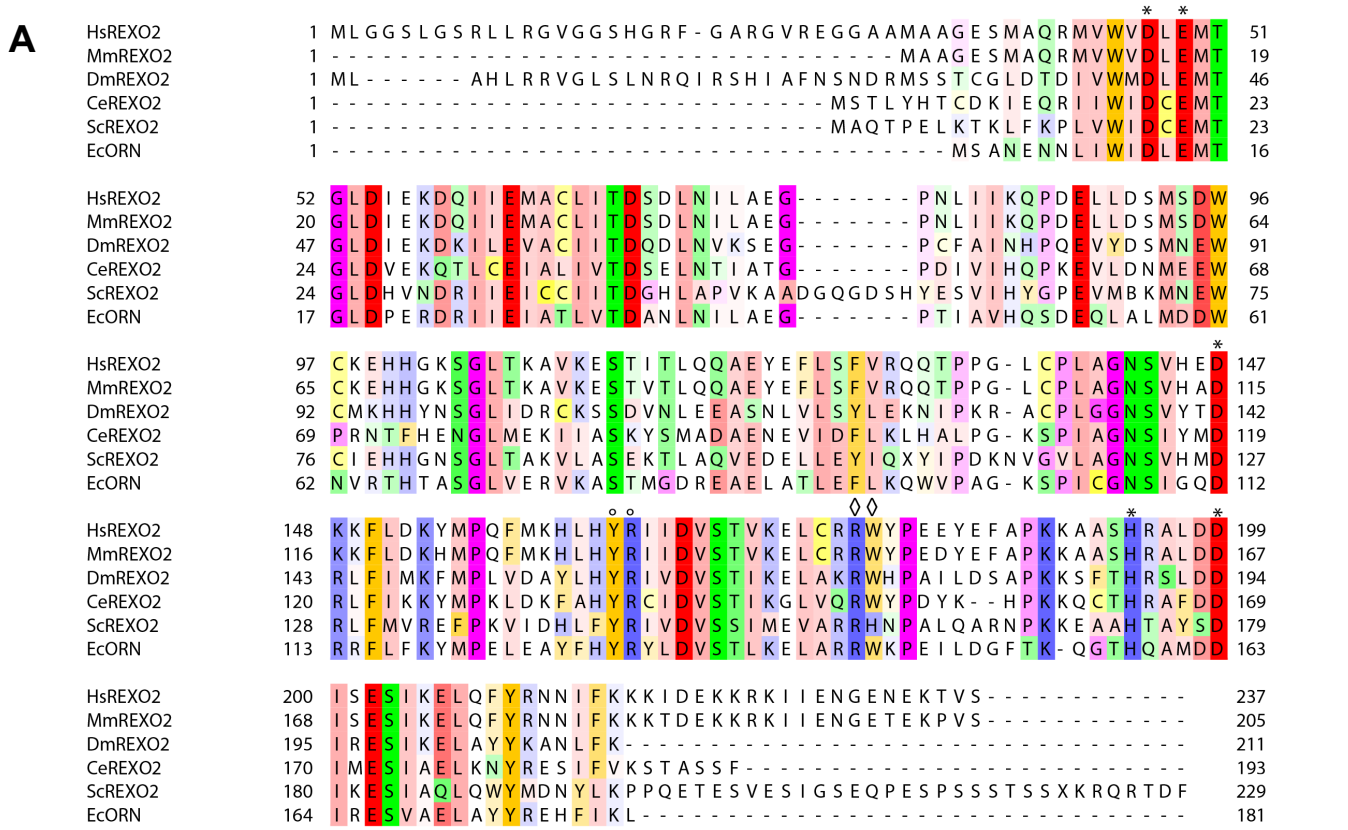
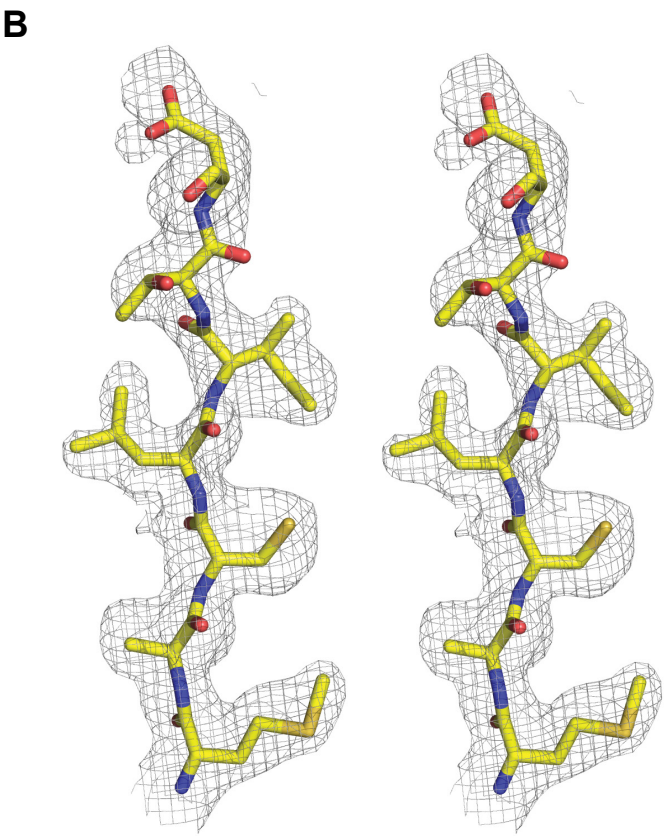


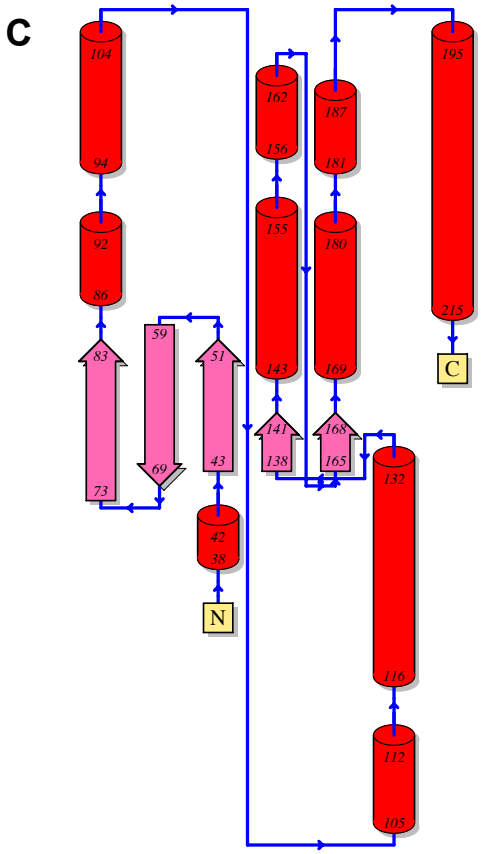
Figure S1, related to Figure 1.

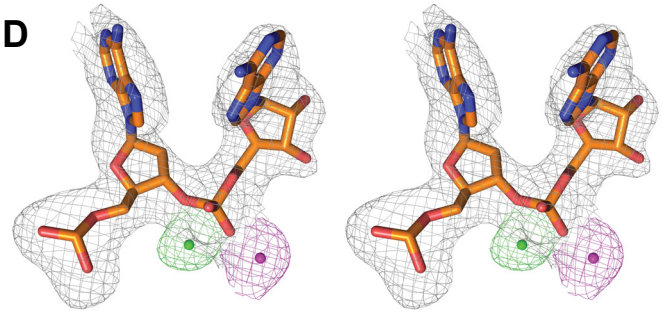
(A) Purified REXO2 variants used for biochemical characterisation and structure determination. Each variant (25 pmol) was separated on a 4-20% Mini-PROTEAN TGX gel (Bio-Rad) and detected by stain-free imaging. **(B)** Activity of wild-type REXO2 upon RNA 3-5-mer substrates, using higher REXO2 concentrations than those in Figure 1. Monophosphorylated radiolabelled substrate (10 fmol) was incubated with the indicated amount of REXO2 at 37°C for 30 min, separated by 18% urea-PAGE and imaged by autoradiography. **(C)** *In vitro* nuclease assays as in (B), using DNA 3-5-mer substrates. **(D)** Quantification of RNA degradation by REXO2 from (B). **(E)** Quantification of DNA degradation by REXO2 from (C). **(F)** Activity of the truncated wild-type REXO2 variant used for crystallisation (comprising amino acids 39-216) upon RNA dinucleotides. Reaction were carried out as in (B). **(G)** *In vitro* nuclease assays as in (F), using DNA dinucleotide substrates. **(H)** Quantification of RNA dinucleotide degradation by the wild-type 39-216 REXO2 as in (F). **(I)** Quantification of DNA dinucleotide degradation by the wild-type 39-216 REXO2 as in (G).

Figure S2, related to Figure 2.









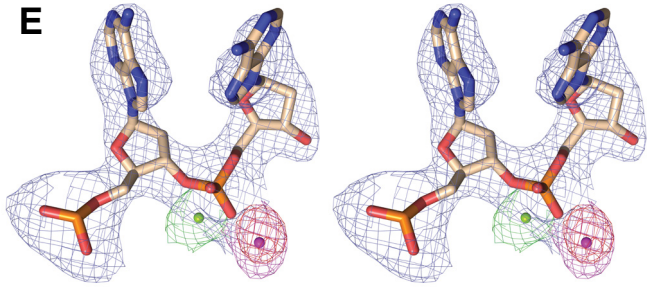


Figure S2, related to Figure 2.

(A) Sequence alignment of REXO2. Sequence conservation is shown with Zappo color code (i.e., aliphatic/ hydrophobic, pink; aromatic, orange; positive, blue; negative, red; hydrophilic, green; conformationally special, magenta; and cysteine, yellow). Species abbreviations are: Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster; Ce, Caenorhabditis elegans; Sc, Saccharomyces cerevisiae; Ec, Escherichia coli.* The alignment was performed using ClustalO (Sievers et al., 2011). DEDDh residues, Y164-R165, and R178-W179 are indicated with asterisks, circles and diamonds, respectively. **(B)** Stereo image showing a F_O-F_C electron density omit map of a representative section of REXO2 contoured at 2.0 σ. Residues are shown as yellow sticks and map in grey mesh. **(C)** Topology diagram of REXO2. α-helices are represented by red cylinders and β-strands by pink arrows. The beginning and end of each secondary structure element is indicated. **(D)** Stereo image showing a F_O-F_C electron density omit map of the pApA RNA dinucleotide (grey mesh), Zn^{2+} (pink mesh), and Mg^{2+} (green mesh) of REXO2 contoured at 2.0, 4.0, and 2.0 σ, respectively. Residues are shown as orange sticks, Zn^{2+} as a pink sphere and Mg^{2+} as a green sphere. **(E)** Stereo image showing a F_O-F_C electron density omit map of the dAdA DNA dinucleotide contoured at the same level as in (D). In addition, an anomalous map of Zn^{2+} is shown in red mesh contoured at 10 σ.

Figure S3, related to Figure 2.

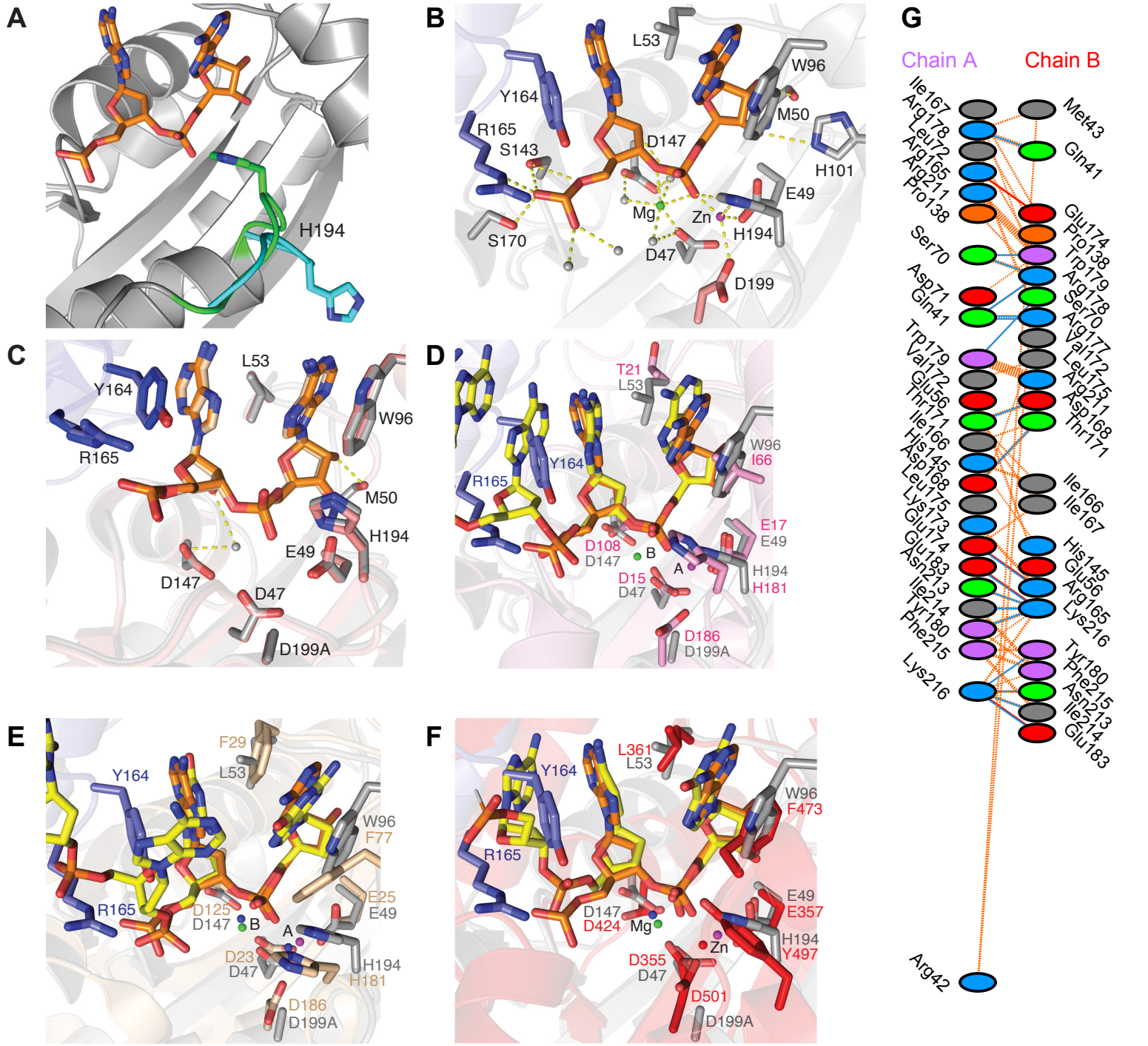
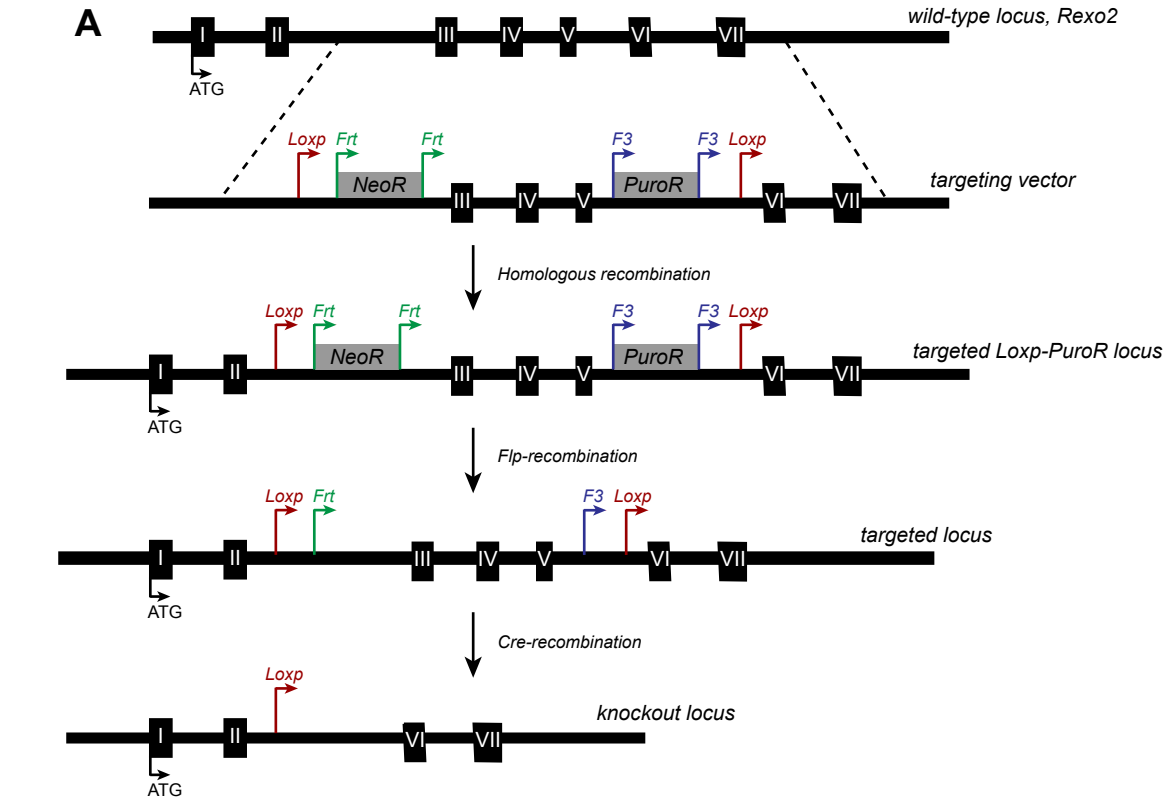


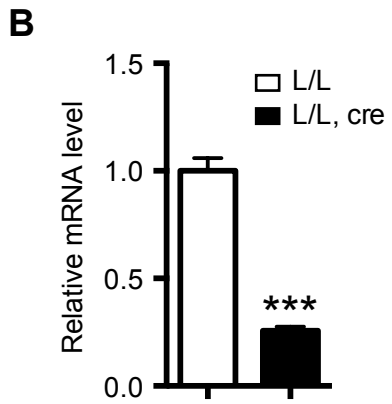
Figure S3, related to Figure 2.

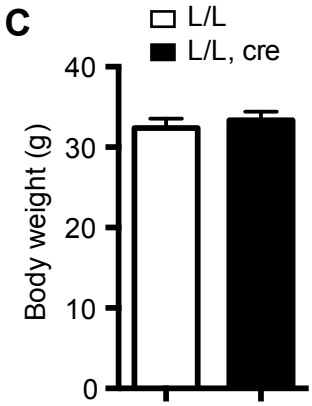
(A) The trigger loop of REXO2 is shown from the apo structure (in cyan) and from the RNA substrate-bound structure (in green). The pApA dinucleotide is shown as orange sticks. **(B)** The REXO2 active site. The pApA dinucleotide (orange) interacting residues are shown with sticks and the polar bonds as yellow dashes. Zn^{2+} and Mg^{2+} are shown as pink and green spheres, respectively. Water molecules are shown as grey spheres. The modelled D199 is shown in pink. **(C)** Alignment of REXO2-pApA and REXO2-dAdA. REXO2-pApA residues use the same colour code as in (B) and REXO2-dAdA is shown in pink with its nucleotides in sand colour. RNA-specific bonds are shown with yellow dashes and a water molecule as a grey sphere. The alignment was performed with the SSM method. **(D)** Alignment of REXO2-pApA and exonuclease I (PDB: 4JS4). REXO2 residues use the same colour code as in (B), and Exonuclease I is shown in pink with its nucleotides in yellow. Metal sites A and B are indicated. **(E)** Alignment of REXO2-pApA and RNase T (PDB: 3V9X). REXO2 residues use the same colour code as (B), and RNase T is shown in sand colour with its nucleotides in yellow. Bound Mg^{2+} ions in RNase T are shown as blue spheres. Metal sites A and B are indicated. **(F)** Alignment of REXO2-pApA and the Klenow fragment of DNA polymerase I (PDB: 1KFS). REXO2 residues use the same colour code as in (B) and DNA polymerase I is shown in red with its nucleotides in yellow. Zn^{2+} and Mg^{2+} in the DNA polymerase I structure are shown as red and blue spheres, respectively. The alignment was performed with the least square fit method using the pApA dinucleotides of REXO2 as reference residue range and the last two 3′ residues of exonuclease I, RNase T or DNA polymerase I as moving residue range. **(G)** The number of H-bond lines between any two residues indicates the number of potential hydrogen bonds between them. For non-bonded contacts, the width of the striped line is proportional to the number of atomic contacts. Residue colors: blue=positive (H,K,R); red=negative (D,E); green=neutral (S,T,N,Q); black=aliphatic (A,V,L,I,M); pink=aromatic (F,Y,W); brown (P,G); light

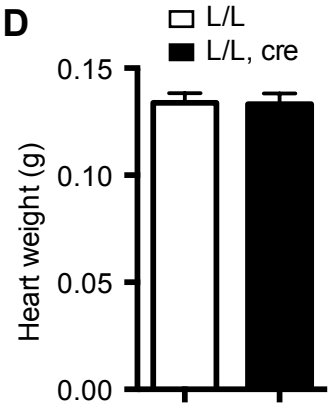
green (C). **(H-K)** Gel filtration profiles of the REXO2 dimer mutants. REXO2 wild-type (H) or mutants (I-K) were subjected to gel filtration on a Superdex 75 increase column and peak fractions separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). REXO2 proteins are indicated with arrows, and contaminating bands are indicated with asterisks.

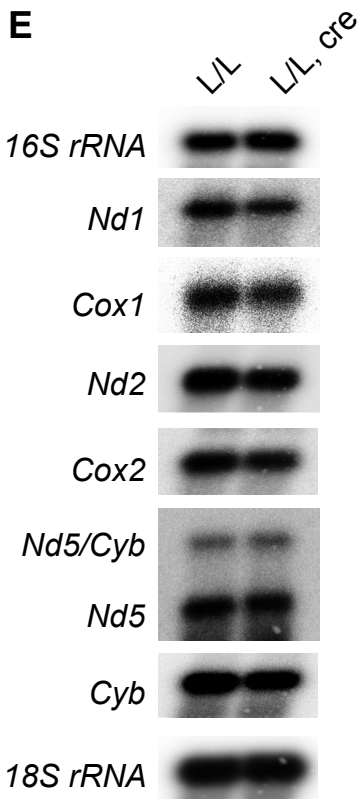
Figure S4, related to Figure 4.











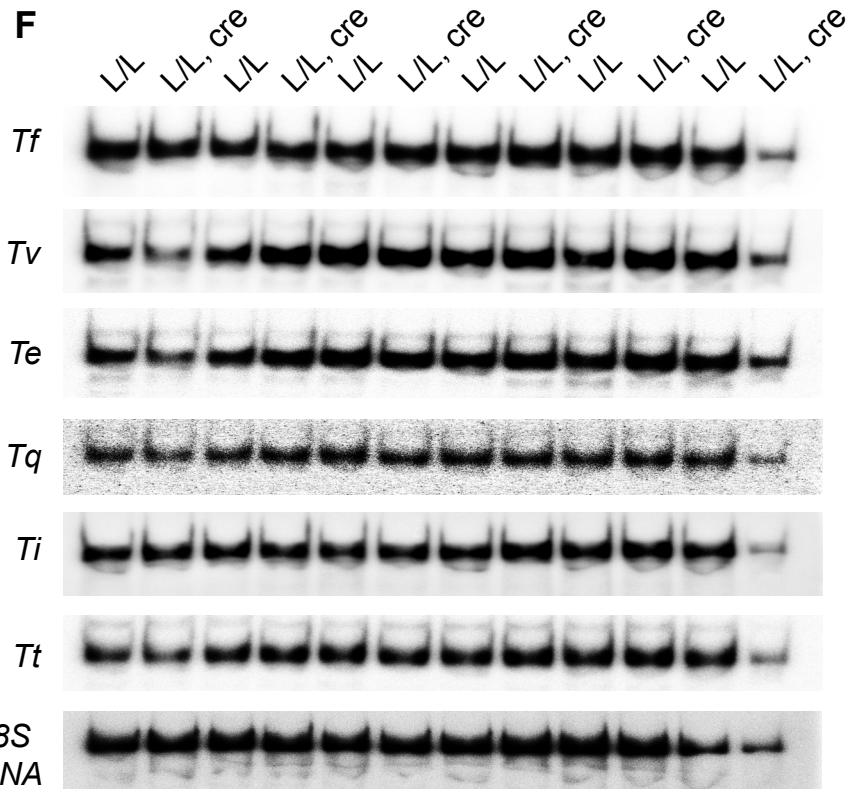


Figure S4, related to Figure 4.

(A) Targeting strategy for knockout of the *Rexo2* gene. **(B)** qPCR analyses of *Rexo2* expression in control (L/L) and *Rexo2* knockout (L,L, cre) mouse hearts. *Rexo2* levels are normalized to the level of *Actin* and represent mean values from 3 independent experiments with total n = 15 mice for each group, error bars represent SEM, ***p < 0.001. **(C, D)** Body weight (C) and heart weight (D) in control (L/L) and *Rexo2* knockout (L/L, cre) mice at 52 weeks old. Total n = 21 (L/L) and n = 23 (L/L, cre) mice, error bars represent SEM. **(E)** Representative northern blot images showing mitochondrial mRNA steady-state levels in control and knockout mice at 52 weeks old. *18S rRNA* is used as a loading control. Northern blots were performed 3 times with total n = 10 mice for each group. **(F)** Representative northern blot images showing steady-state levels of mitochondrial tRNA (single letter code) in control (L/L) and *Rexo2* knockout (L/L, cre) mice at 52 weeks old. *5.8S rRNA* was used as a loading control. Northern blots were performed 3 times with total n = 15 mice for each group.

Figure S5, related to Figure 5.

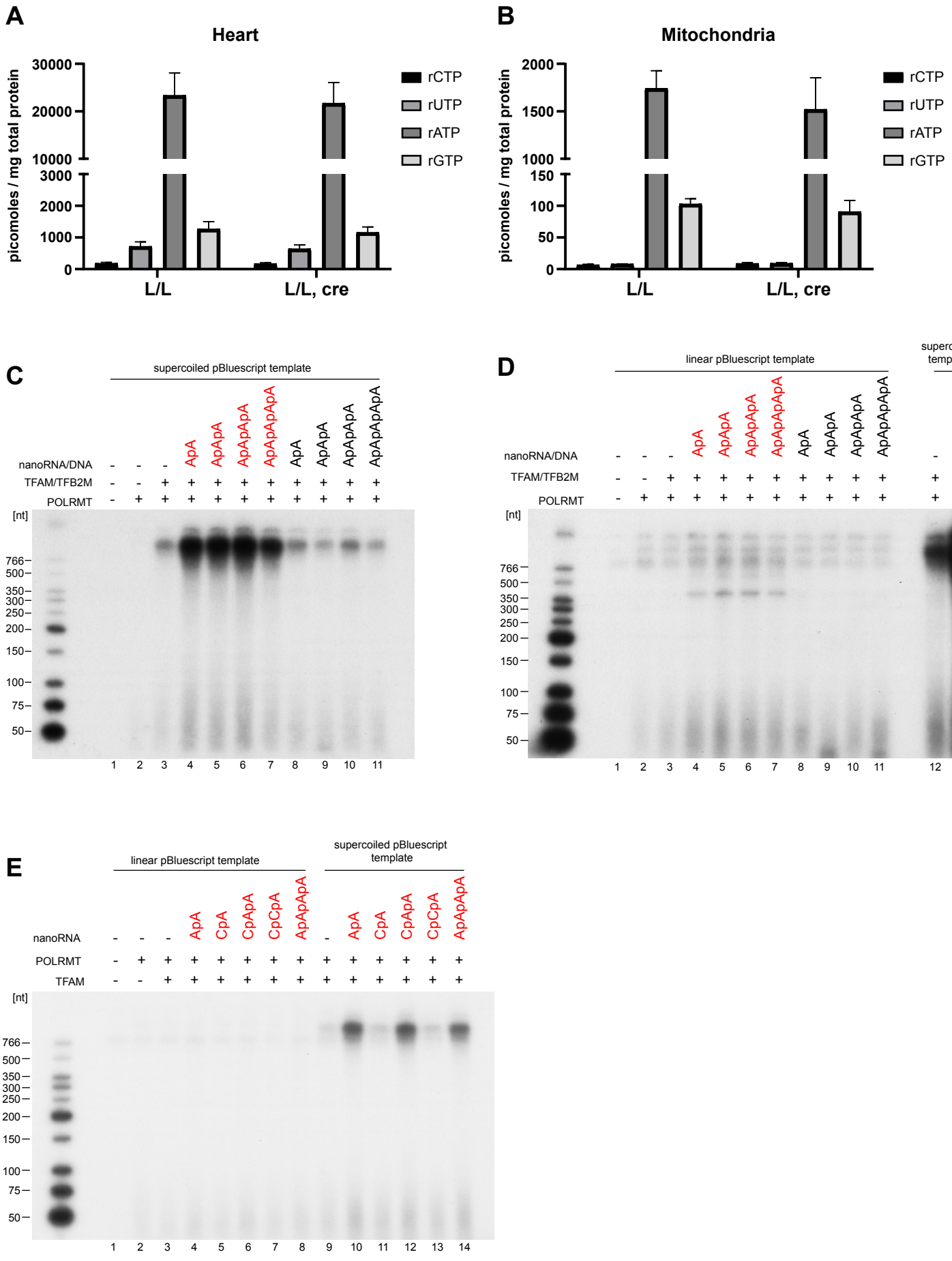


Figure S5, related to Figure 5.

(A, B) Quantification of nucleotide concentrations in heart tissue (A) or isolated mitochondria (B) of wild-type (L/L) or *Rexo2* knockout (L/L, cre) mice determined using HPLC. Bars indicate mean values from three mice in each group, error bars represent SEM. **(C)** *In vitro* transcription reactions containing supercoiled pBluescript II SK (+) DNA as a template, 100 μM each ATP, CTP and GTP, 1 μM UTP and 0.2 μCi ^{32}P α-UTP, and 20 μM poly(A) RNA (red) or DNA (black) oligonucleotides where indicated. **(D)** *In vitro* transcription reactions as in (C), but using linearised pBluescript II SK(+) DNA as a template. Reactions using a supercoiled template (lanes 12 and 13) are included as a positive control. **(E)** *In vitro* transcription reactions containing linearised or supercoiled pBluescript II SK(+) template, 20 μM each ATP, CTP and GTP, 1 μM UTP and 0.2 μCi ^{32}P α-UTP and 20 μM RNA oligonucleotides of different sequences as indicated.

Figure S6, related to Figure 6.

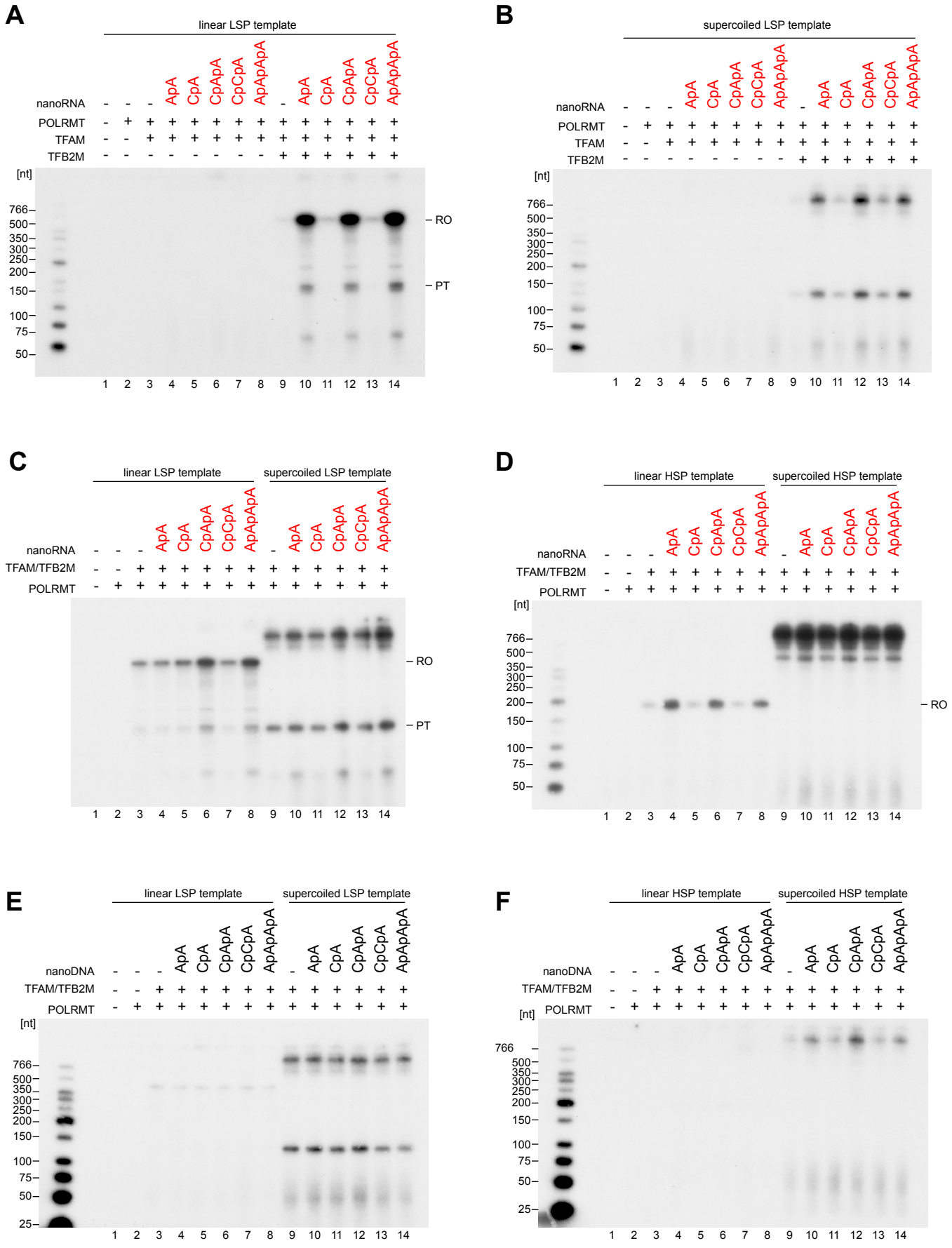
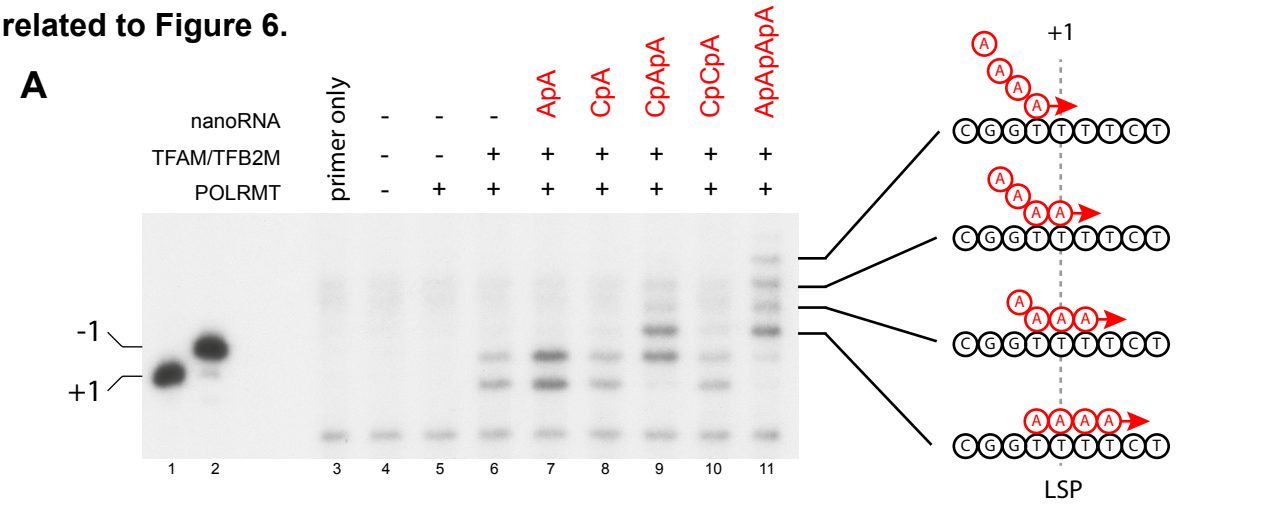
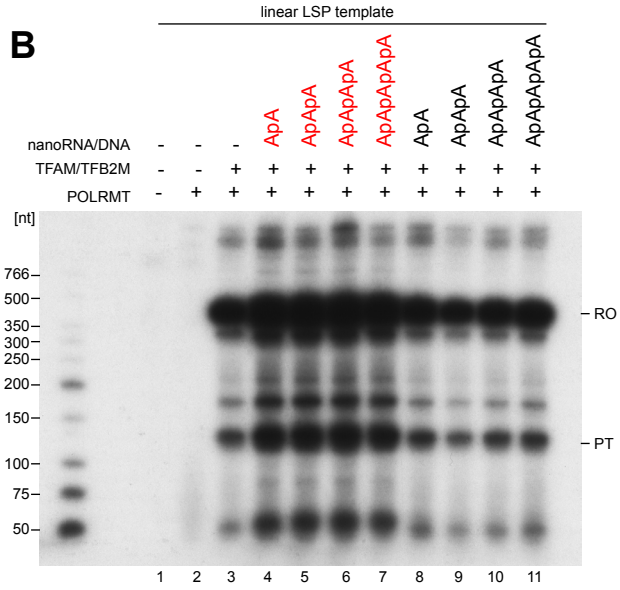


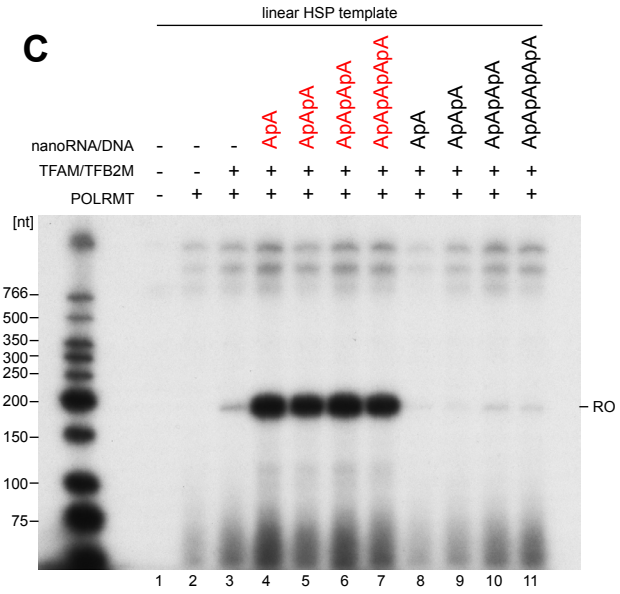
Figure S6, related to Figure 6.

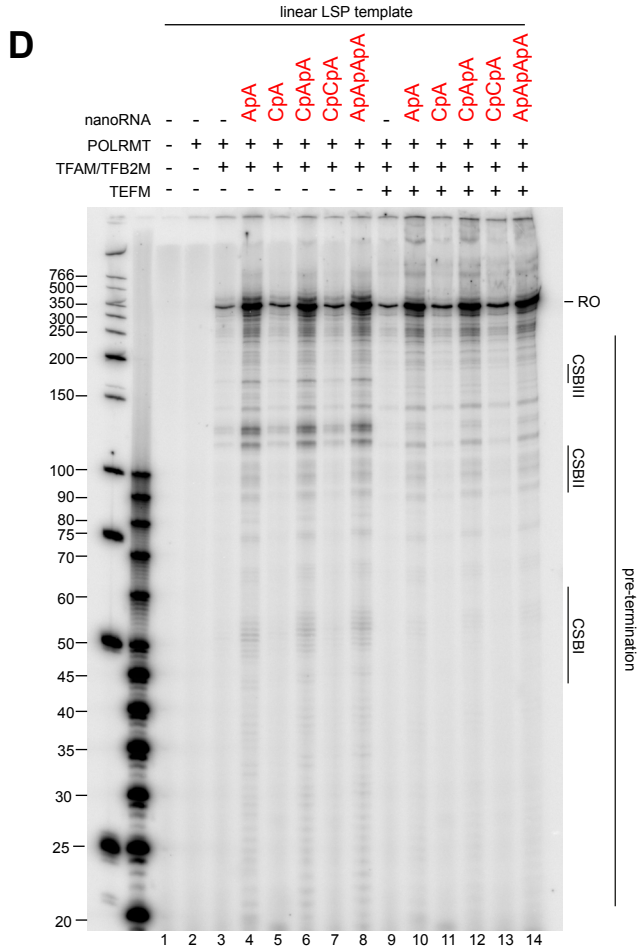
(A) *In vitro* transcription reactions using a linearised LSP-containing template in the presence or absence of TFB2M. Reactions contain 20 μM each ATP, CTP and GTP, 1 μM UTP and 0.2 μCi ^{32}P α-UTP, and 20 μM of the indicated RNA oligonucleotides. **(B)** *In vitro* transcription reactions as in (A) but using a supercoiled LSP-containing template. **(C)** *In vitro* transcription reactions using a linearized or supercoiled LSP-containing template, 100 μM each ATP, CTP and GTP, 1 μM UTP and 0.2 μCi ^{32}P α-UTP, and 20 μM of the indicated RNA oligonucleotides. **(D)** *In vitro* transcription reactions as in (C) but using linearised or supercoiled HSP-containing templates. **(E)** *In vitro* transcription reactions as in (C) but with the addition of DNA oligonucleotides instead of RNA oligonucleotides. **(F)** *In vitro* transcription reactions as in (D) but with the addition of DNA oligonucleotides instead of RNA oligonucleotides.

Figure S7, related to Figure 6.









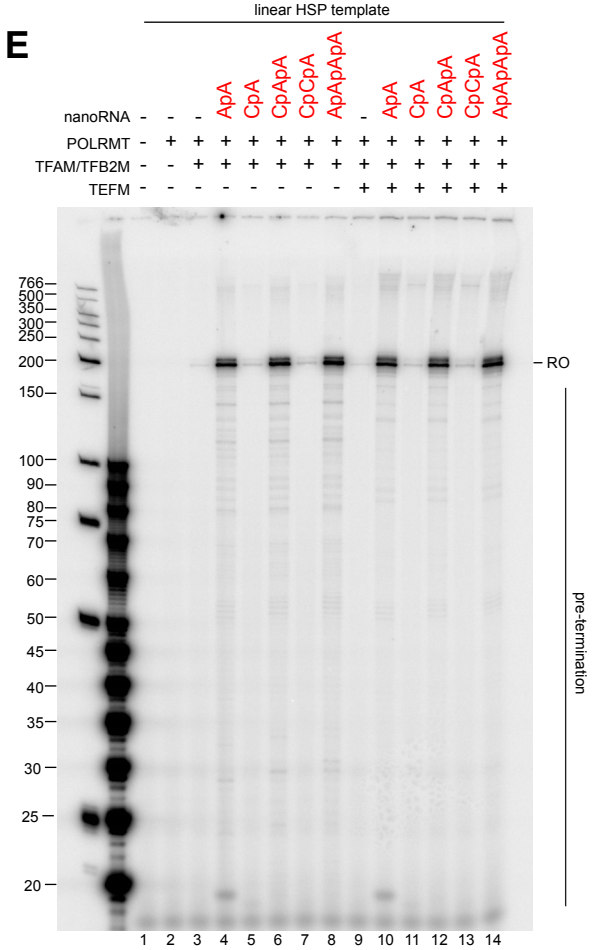


Figure S7, related to Figure 6.

(A) Primer extension analysis of nanoRNA-primed transcription reactions. Transcription was carried out in the absence of a radiolabel, then a 5' radiolabelled primer was annealed and extended to map the 5' sites of transcription products. Labelled synthesised oligos in lanes 1 and 2 indicate the migration of transcription products initiated from the -1 and +1 sites, respectively. **(B)** *In vitro* transcription reactions using a linearized LSP-containing template, 100 μM each ATP, CTP and GTP, 1 μM UTP and 0.2 μCi ^{32}P α-UTP, and 20 μM of the indicated poly(A) RNA (red) or DNA (black) oligonucleotides. **(C)** *In vitro* transcription reactions as in (B) but using a linearized HSP-containing template. **(D)** nanoRNA-primed *in vitro* transcription reactions using a linearized LSP template, in the absence or presence of TEFM. 'RO' indicates the runoff transcription product, and CSBI, CSBII and CSBIII indicate the locations of conserved sequence boxes. Transcription products shorter than the expected runoff transcript are designated 'pre-termination'. **(E)** *In vitro* transcription reactions as in (D) but using a linearized HSP-containing template in the absence or presence of TEFM.

Table S1, related to Figure 2. Structural homologues of REXO2 using Dali.

Chain	Z	rmsd	lali	nres	% id	Protein
1j9a-A	27.0	1.3	178	184	49	Oligoribonuclease
4js4-A	16.6	2.7	168	468	14	Exonuclease I
3v9x-B	14.9	3.0	162	212	13	RNase T
4hec-B	14.9	2.7	153	163	13	Putative uncharacterized protein
5yws-B	13.8	2.8	161	234	16	Three-prime repair Exonuclease I
5l80-A	13.1	2.6	148	301	12	Maternal protein Exu
3cg7-A	12.7	2.7	161	296	15	Cell death-related nuclease 4
1zbu-B	12.2	2.9	156	304	15	3'-5' exonuclease Eri1
5m1s-D	11.8	3.0	153	229	16	Dna polymerase iii subunit alpha
4b8c-A	11.2	3.2	159	267	12	Poly(a) ribonuclease Pop2
2a1s-C	10.7	3.4	154	393	10	Poly(a)-specific ribonuclease Parn

Table S3, related to STAR methods. Sequences for oligonucleotides used.

Taqman assays and oligonucleotides		
Transcript	**Source**	**Identifier**
12S	Thermo Fisher Scientific	Mm04260177_s1
16S	Thermo Fisher Scientific	Mm04260181_s1
Actin	Thermo Fisher Scientific	Mm01205647_g1
Atp6	Thermo Fisher Scientific	Mm03649417_g1
Atp8	Thermo Fisher Scientific	Mm04225236_g1
Cox1	Thermo Fisher Scientific	Mm04225243_g1
Cox2	Thermo Fisher Scientific	Mm03294838_g1
Cox3	Thermo Fisher Scientific	Mm04225261_g1
Cyb	Thermo Fisher Scientific	Mm04225271_g1
Nd1	Thermo Fisher Scientific	Mm04225274_g1
Nd2	Thermo Fisher Scientific	Mm04225288_s1
Nd3	Thermo Fisher Scientific	Mm04225292_g1
Nd4l/4	Thermo Fisher Scientific	Mm04225294_s1
Nd5	Thermo Fisher Scientific	Custom made_AIHSNT9
Nd6	Thermo Fisher Scientific	Custom made_AIVI3E8
Tefm	Thermo Fisher Scientific	Mm01304209_m1
RNA oligonucleotides for nuclease and transcription assays		
Length	**Sequences**	
2-mer	AA, CC, GG, UU, CA	
3-mer	CAA, CCA, AAA, GGG	
4-mer	AAAA	
5-mer	AAAAA	
DNA oligonucleotides for nuclease and transcription assays		
Length	**Sequences**	
2-mer	AA, CC, GG, UU, CA	
3-mer	CAA, CCA, AAA, GGG	
4-mer	AAAA	
5-mer	AAAAA	

Table S3 (cont.)

Oligonucleotides for genotyping	
Name	**Sequence**
Cre-F	CACGACCAAGTGACAGCAAT
Cre-R	AGAGACGGAAATCCATCGCT
Rexo2 primer 29-F	GTCTAGGTGATGGCGTCAGTC
Rexo2 primer 30-F	CTTCCTCTGTGGTGTTCTTCC
Primers for SYBR Green qPCR	
Name	**Sequence**
Actin-F	GGCTGTATTCCCCTCCATCG
Actin-R	CCAGTTGGTAACAATGCCATGT
Rexo2-exon4-F	AACTCAGTTCATGCAGATAAG
Rexo2-exon4-F	TGCACAGCTCTTTAACAGTG
Probes for tRNA northern blots	
Name	**Sequence**
mt-tRNA^{F}	CATTTTCAGTGCTTTGCTTTGTTATTA
mt-tRNA^{V}	GTGTAGGCCAGATGCTTTAAT
mt-tRNA^{E}	AACTGCGACCAATGACATGAAAAATC
mt-tRNA^{Q}	TTCAAAATTCTCCGTGCTACCTAAACA
mt-tRNA^{T}	AAGATCTTCATTTCAGGTTTACAA
Oligo for primer extension	
Name	**Sequence**
hLSP_PEx	CACCAGCCTAACCAGATTTC