Supplementary Figures and Legends

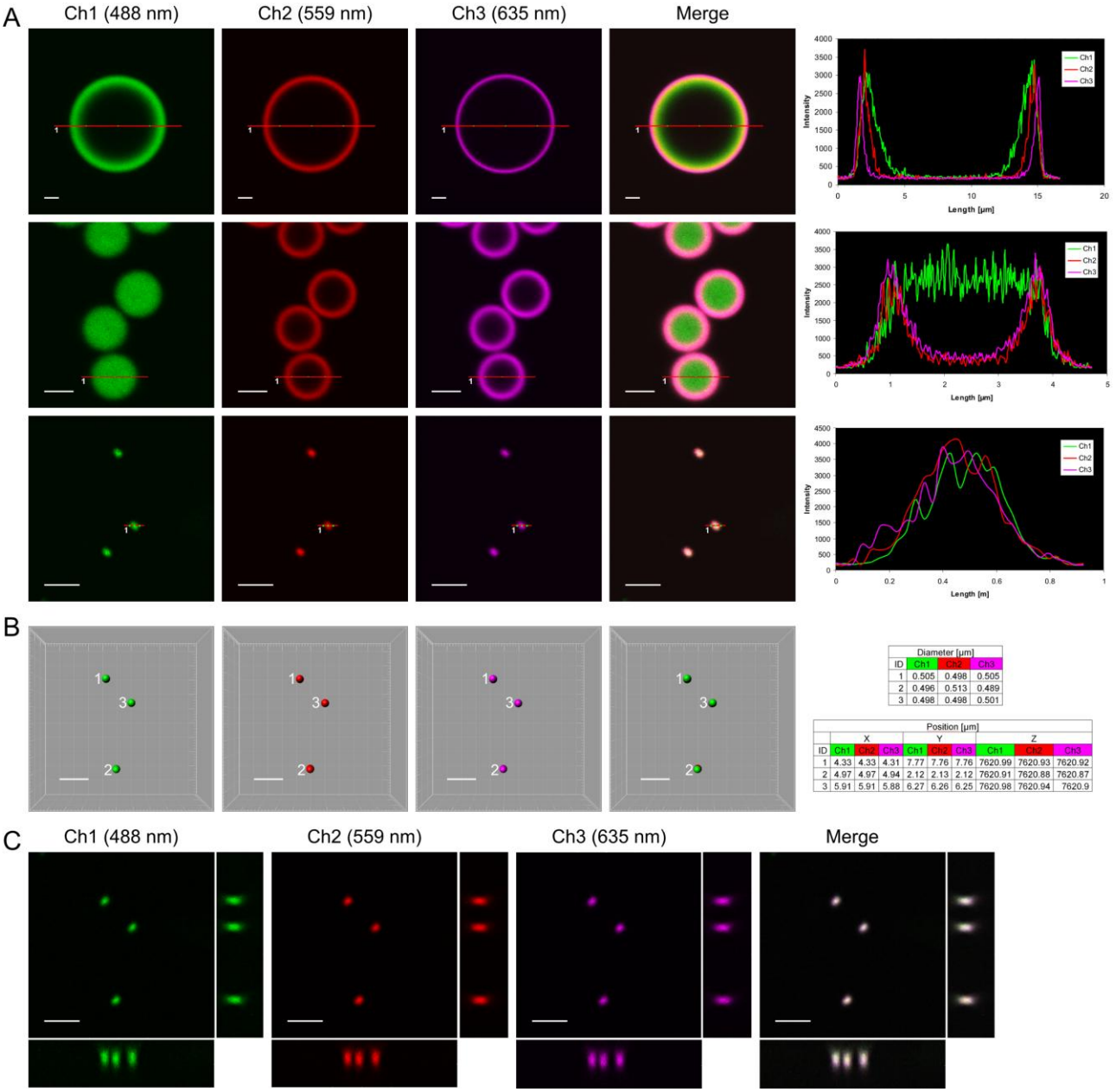

Figure S1. Tests of the optical alignment and focal properties of the confocal microscope. (A) Images of the optical cross-section of fluorescent microspheres differing in diameter. Each microsphere was scanned in 3 different fluorescence channels. Fluorescence intensity profiles for each channel are presented in graphs on the right of the images. (B) 3D reconstruction of the Z-stack images of the smallest fluorescent microspheres. Spheres were reconstructed in each channel and merged. The diameter and XYZ position of each reconstructed microsphere are presented in tables. (C) Presentation of Z-axis alignment. Z-stack images (used in 3D reconstruction in panel B) are shown. White bars in all images represent 2µm.

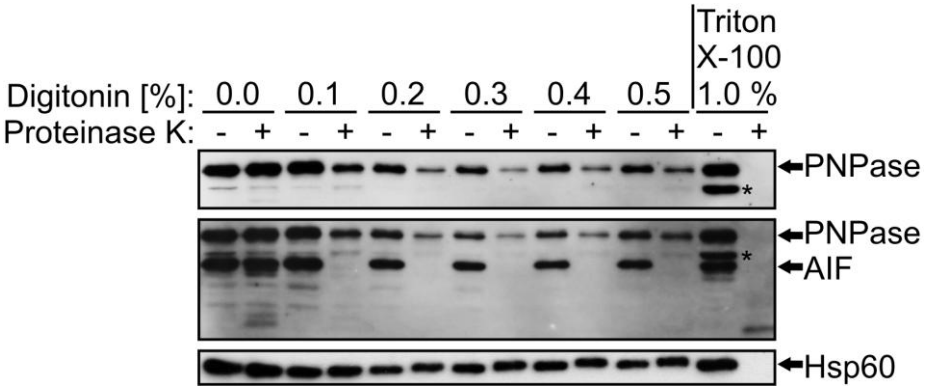

Figure S2. A fraction of PNPase is localized in the mitochondrial matrix. Mitochondria isolated from 293 T-Rex cells were treated with increasing concentrations of digitonin in order to disrupt the outer mitochondrial membrane. Obtained mitoplasts were treated with proteinase K to remove the intermembrane space proteins. Subsequently mitoplasts were lysed and analyzed by western blot using antibodies against PNPase, AIF (intermembrane space marker) and Hsp60 (matrix marker). The same membrane was first probed for PNPase and then for AIF. Asterisk indicates degradation product of PNPase.

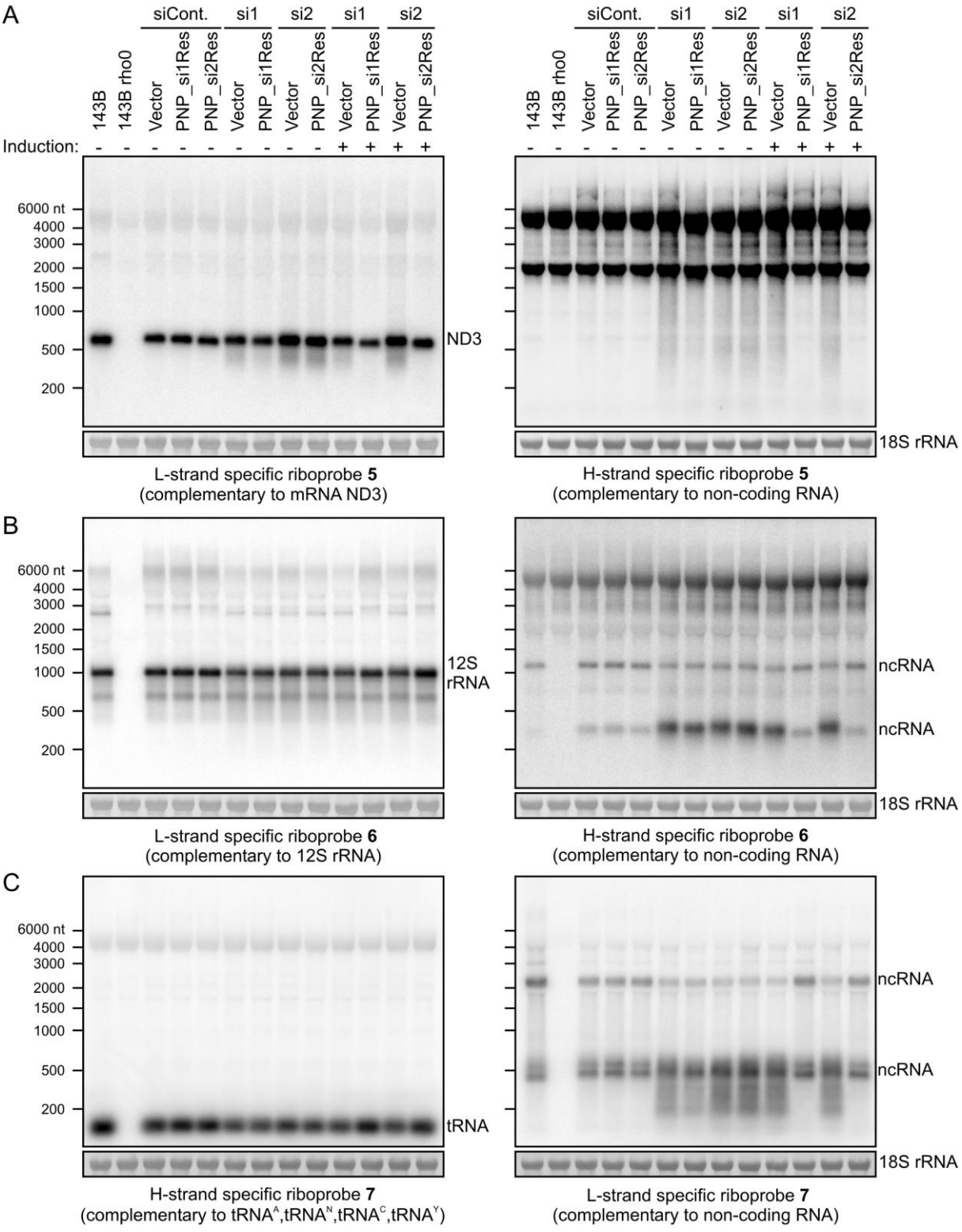
A
siCont.
si1
si2
si1
si2
143B
143B rho0
Vector
PNP_si1Res
PNP_si2Res
Induction:
6000 nt
4000
3000
2000
1500
1000
500
200
ND3
18S rRNA
L-strand specific riboprobe 5
(complementary to mRNA ND3)
H-strand specific riboprobe 5
(complementary to non-coding RNA)
B
12S
rRNA
ncRNA
ncRNA
L-strand specific riboprobe 6
(complementary to 12S rRNA)
H-strand specific riboprobe 6
(complementary to non-coding RNA)
C
tRNA
H-strand specific riboprobe 7
(complementary to tRNA^A,tRNA^N,tRNA^C,tRNA^Y)
L-strand specific riboprobe 7
(complementary to non-coding RNA)

Figure S3

Figure S3. PNPase depletion affects mitochondrial mRNA, rRNA, and tRNA metabolism. (A, B, C) Northern blot analysis of RNA isolated from cells treated exactly the same as in Fig. 2. Strand specific riboprobes were used. Expression of exogenous PNPase was induced 4 days prior to cell collection. Some hybridization signal was unspecific (especially from cytoplasmic rRNA). This was controlled using RNA isolated from 143B rho^0 and 143B cells. Cytosolic 18S rRNA staining by methylene blue is shown as a loading control.

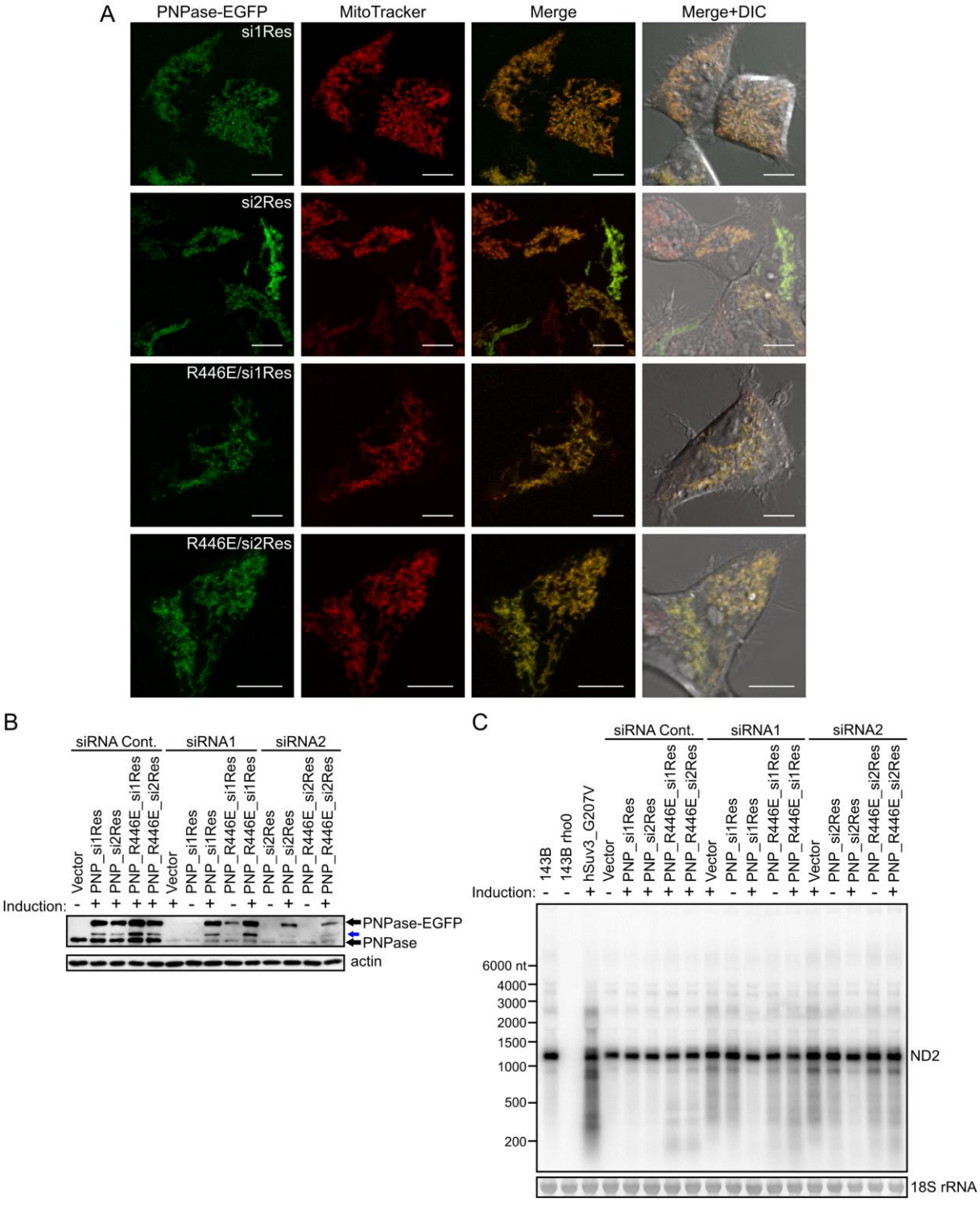
A
PNPase-EGFP
MitoTracker
Merge
Merge+DIC
si1Res
si2Res
R446E/si1Res
R446E/si2Res
B
siRNA Cont.
siRNA1
siRNA2
Induction:
Vector
PNP_si1Res
PNP_si2Res
PNP_R446E_si1Res
PNP_R446E_si2Res
PNPase-EGFP
PNPase
actin
C
143B
143B rho0
hSuv3_G207V
6000 nt
4000
3000
2000
1500
1000
500
200
ND2
18S rRNA

Figure S4

Figure S4. EGFP-tagged PNPase is capable of replacing the endogenous protein in mtRNA decay. (A) Live imaging of stable 293 cell lines expressing C-terminally EGFP tagged wild type or inactive PNPase (R446E), resistant to siRNAs si1 and si2, which are specific for the endogenous PNPase. Mitochondria were labeled with MitoTracker Orange. A merge with a view of differential interference contrast (DIC) is shown. Scale bars represent 10 μm. (B, C) The same stable cell lines were transfected with two different siRNAs (si1, si2) to deplete endogenous PNPase and simultaneously treated with tetracycline to induce expression of catalytically active PNPase resistant to siRNA (PNP_si1Res, PNP_si2Res) or its inactive counterpart (PNP_R446E_si1Res, PNP_R446E_si2Res). As a control, a stable cell line with integrated empty vector was used and also transfection with control siRNA was performed. The 293 cell line expressing catalytically inactive hSuv3 helicase (hSuv3_G207V) was used as a positive control for the effects of PNPase silencing on mtRNA metabolism. Cells were collected after 4 days of siRNA treatment and analyzed by western and northern techniques. (B) Western blot analysis of the endogenous PNPase levels and its EGFP tagged forms using antibodies raised against PNPase. For standardization, actin staining was performed. Blue arrow indicates degradation product of EGFP tagged proteins. (C) Northern blot analysis of ND2. Probe detecting RNA transcribed from both H and L-strand of mtDNA was used. Cytosolic 18S rRNA staining by methylene blue is shown as a loading control. Expression of catalytically active PNPase-EGFP but not its inactive form restores mtRNA decay affected by endogenous PNPase depletion which is manifested by disappearing of degradation intermediates. The presence of mutated PNPase^{R446E}-EGFP alone induces dominant negative effect, although to weaker extent than expression of inactive hSuv3 helicase.

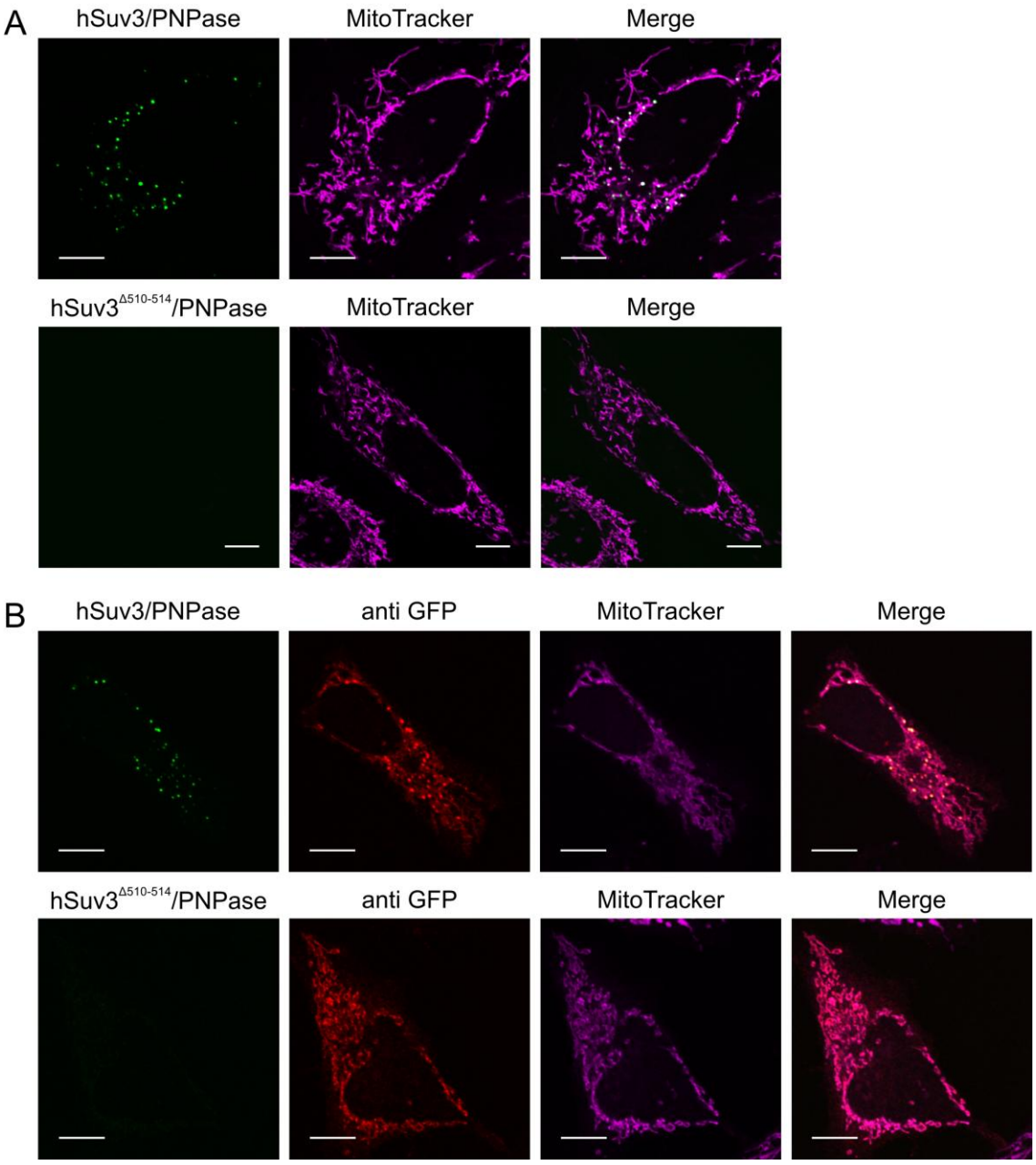
A
hSuv3/PNPase
MitoTracker
Merge
hSuv3Δ510-514/PNPase
MitoTracker
Merge
B
hSuv3/PNPase
anti GFP
MitoTracker
Merge
hSuv3Δ510-514/PNPase
anti GFP
MitoTracker
Merge

Figure S5

Figure S5. Expression of hSuv3$^{\Delta 510\text{-}514}$/PNPase BiFC pair does not result in fluorescence complementation. (A) Images of living cells transfected with a mixture of plasmids encoding wild type forms of PNPase and hSuv3 (upper panel) or wild type PNPase and hSuv3$^{\Delta 510\text{-}514}$ (lower panel). PNPase was expressed in fusion with the N-terminal part of Venus, whereas hSuv3 variants were expressed in fusion with the C-terminal part of Venus. Mitochondria were stained with MitoTracker Orange. Both image panels were acquired with the same microscope settings. Deletion of residue 510-514 in hSuv3 inhibits fluorescence complementation. (B) Immunostaining of hSuv3-C-Venus (upper panel) and hSuv3$^{\Delta 510\text{-}514}$-C-Venus (lower panel). Cells were transfected with the same mixtures of plasmids as in panel A. Immunostaining was carried out using anti-GFP antibodies detecting the C-terminal part of Venus and mitochondria were stained with MitoTracker DeepRed. Images on both panels were acquired at the same microscope settings. Deletion of residue 510-514 in hSuv3 inhibits fluorescent complementation although this protein is expressed at levels similar to the full length one. (A, B) White bars represent 10μm.

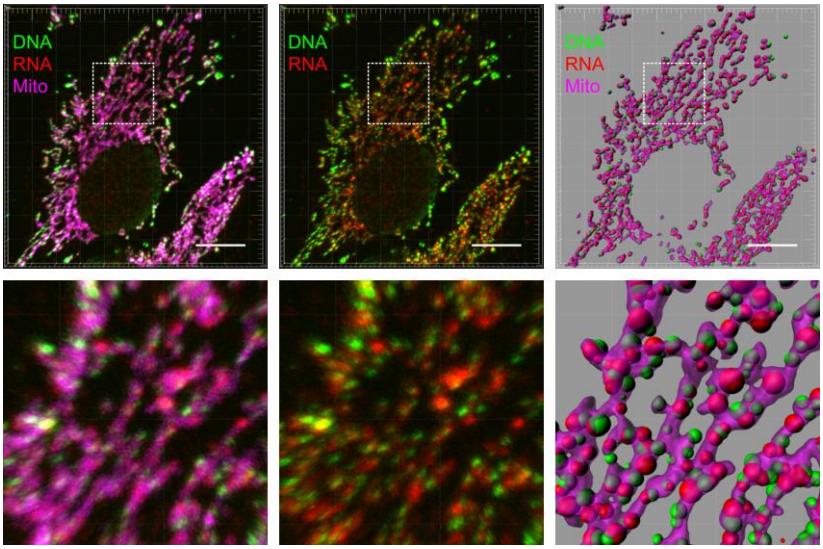

Figure S6. Detection of mitochondrial RNA and mitochondrial DNA. Images of 143B cells incubated with BrU and immunostained with anti-BrU (RNA) and anti-DNA antibodies. Mitochondria were labeled with MitoTracker DeepRed. 3D reconstruction images are shown. Zoomed images are presented on the lower panel. The surface of mitochondria in 3D reconstructions was made transparent to visualize mtRNA and mtDNA.

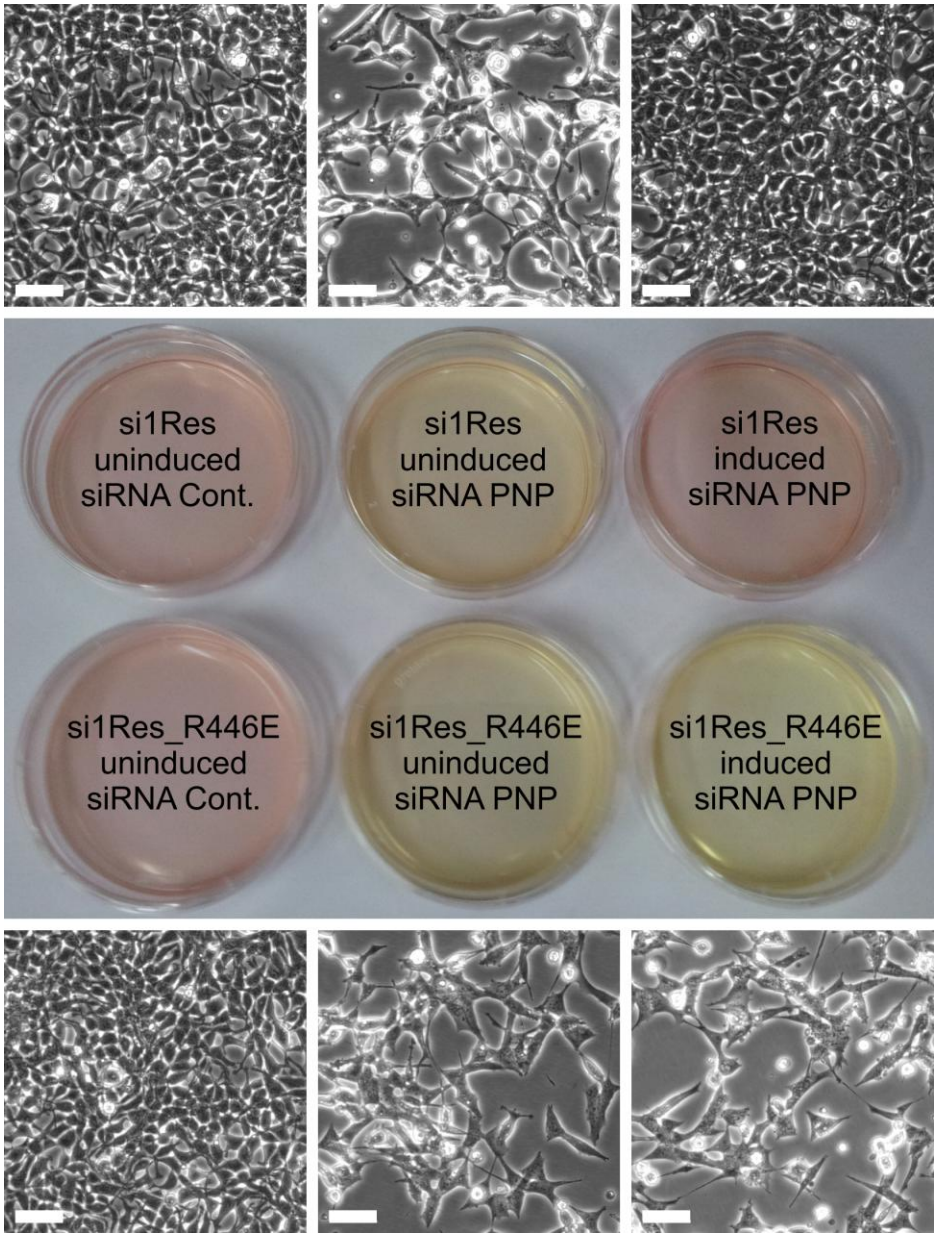

Figure S7. PNPase depletion affects cells morphology and results in acidification of culture medium. Stable 293 cell lines which express inducible wild-type PNPase resistant to applied siRNA (si1Res) or its inactive counterpart (si1Res_R446E) were transfected with control siRNA or PNPase-specific one (si1). Expression of recoded PNPase was induced at the time of transfecion. Dishes and cells were photographed 8 days after transfecion. Yellow color of the culture medium indicates its acidification. White bars represent 50μm.

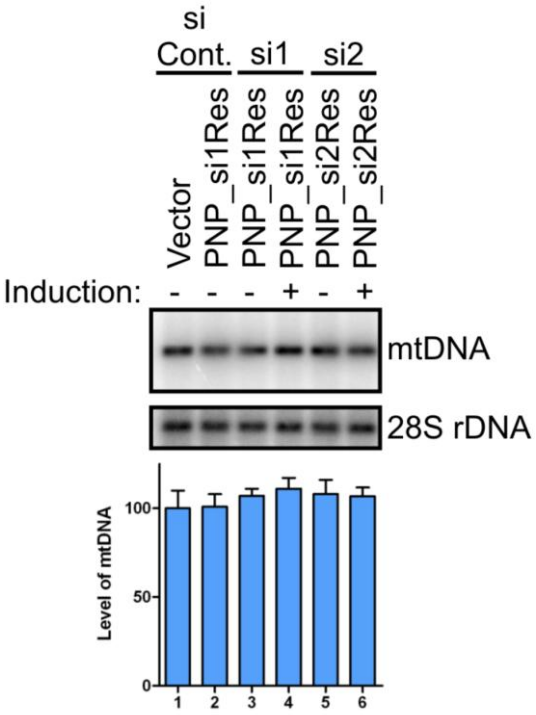

Figure S8. Silencing of PNPase does not affect the levels of mitochondrial DNA. Stable cell lines (PNP_si1Res, PNP_si2Res) which express inducible PNPase resistant to siRNA anti-PNPase or the control cell line with empty vector integrated were transfected with two different siRNAs specific for PNPase (si1, si2) or control siRNA. Total DNA was isolated 4 days after transfection and analyzed by the Southern technique. Mitochondrial DNA was detected using a radioactive probe complementary to the *ND2* gene. Hybridization with 28S rDNA was used as reference. The graph presents the mean value of two independent experiments.

Supplementary Movie Legends

Movie S1. PNPase-EGFP fusion protein is localized in mitochondria. Live imaging of stable 293 cell line inducibly expressing C-terminally EGFP tagged wild type PNPase (green) resistant to siRNA si1 specific for the endogenous PNPase. Mitochondria were labeled with MitoTracker Orange (red). A merge with a view of differential interference contrast (DIC) is shown. Expression of PNPase was induced by addition of tetracycline to the culture medium.

Movie S2. PNPase-EGFP fusion protein is expressed in inducible manner. Live imaging of stable 293 cell line capable of inducible expression of C-terminally EGFP tagged wild type PNPase resistant to siRNA si2 specific for the endogenous PNPase (see Fig. S5A). Expression of PNPase was not induced. Imaging was performed with the same microscope settings as in Movie S1. Mitochondria were labeled with MitoTracker Orange (red). A merge with a view of differential interference contrast (DIC) is shown.

Movie S3. Interaction of PNPase with hSuv3 helicase in the living 143B cell. Live imaging of a 143B osteosarcoma cell co-expressing PNPase and hSuv3 in a fusion with the C- and N-terminal part of the Venus protein, respectively, which results in fluorescence complementation (green spots) in mitochondria. Mitochondria were stained using MitoTracker Orange (red).

Movie S4. Interaction of PNPase with hSuv3 helicase in the living HeLa cell. Live imaging of a HeLa cell co-expressing PNPase and hSuv3 in a fusion with the C- and N-terminal part of the Venus protein, respectively, which results in fluorescence complementation (green spots) in mitochondria. Mitochondria were stained using MitoTracker Orange (red).

Movie S5. The complex of PNPase and hSuv3 is localized inside mitochondria. The results of BiFC studies in HeLa cells are shown. During first 10 seconds of the Movie, the maximum intensity projection of fluorescent signal from mitochondria and PNPase-hSuv3 complex (green spots) is shown. Subsequently, fluorescence signal was used in a 3D rendering of the mitochondrial surface, and D-foci are presented as spheres. Cells were transfected with constructs coding PNPase-C-Venus and hSuv3-N-Venus. Mitochondria were stained using MitoTracker Orange. 3D rendering was done with Imaris software.

Supplementary Methods

DNA cloning and establishment of stably transfected cell lines

To get the pRS289 construct, encoding a C-terminal fusion of PNPase with the FLAG tag, a DNA fragment encoding PNPase was amplified in a polymerase chain reaction (PCR) by using cDNA from 293 T-Rex cells as a template, subcloned into pRS307 (pDriveKan), and cloned into pRS201 (pcDNA5/FRT/TO_FLAG) inframe with the FLAG encoding sequence. Site-directed mutagenesis of pRS289 was performed to obtain construct pRS292 encoding PNPase with a substitution of Arg446 for Glu. In order to generate plasmids encoding PNPase or PNPase_R446E carrying silent mutations in sequences complementary to stealthRNA siPN1 or siPN2, site-directed mutagenesis of pRS289 or pRS292 was performed to obtain of pRS448 (PNPase_si1Res), pRS449 (PNPase_si2Res), pRS501 (PNPase_R446E_si1Res), pRS502 (PNPase_R446E_si2Res). A DNA fragment encoding EGFP was amplified in PCR by using pEGFP-N1 plasmid (Clontech) as a template, and cloned into pRS448, pRS449, pRS501, and pRS502 in place of the FLAG tag encoding sequence. The following plasmids were obtained: pRS510, pRS511, pRS513, and pRS514 respectively. A DNA construct encoding hSuv3 in fusion with the TAP tag (pRS265) was obtained by cloning of a DNA fragment encoding hSuv3 into the pcDNA5/FRT/TO_TAP vector. Site-directed mutagenesis of pRS265 was performed to construct a DNA plasmid encoding hSuv3 fused to the TAP tag and carrying deletion of residues 510-514 (pRS279). In order to obtain constructs encoding hSuv3 with amino acids 510-514 deleted (pRS281) and catalytically inactive hSuv3^{G207V} with the same deletion (pRS282), site-directed mutagenesis of pRS97 and pRS98 was performed, respectively.

Plasmids used for FLIM-FRET experiments were constructed by cloning a DNA fragment encoding hSuv3 into pmCherry-N1 (Clontech) and PNPase into pEGFP-N1 (Clontech) to create fusions with fluorescent proteins at the C-terminus. These DNA constructs were named pRS472 and pRS473, respectively. To generate constructs for BiFC analysis, DNA fragments encoding hSuv3, hSuv3$^{\Delta 510\text{-}514}$ or PNPase were cloned into both vectors pBiFC-VN173 and pBiFC-VC155 (Addgene ID 22010 and 22011, respectively). A DNA fragment encoding an N-terminal fusion of the FLAG tag was removed from vectors by PCR. Plasmids obtained are: pRS407 (hSuv3_VN173), pRS408 (hSuv3_Δ510-514_VN173), pRS409 (hSuv3_VC155), pRS410 (hSuv3_Δ510-514_VC155), pRS411 (PNPase_VN173), and pRS412 (PNPase_VC155). Site-directed mutagenesis was performed on constructs pRS411 and pRS409 to generate constructs encoding PNPase_R446E_VN173 (pRS504) and

hSuv3_G207V_VC155 (pRS415) respectively. All constructs are listed in Table S1. The stable, inducible cell lines were established using the Flp-In^{TM} T-Rex 293 system (Invitrogen) as described previously (Szczesny et al., 2010) and generated plasmids: pRS289, pRS292, pRS448, pRS449, pRS501, pRS502, pRS510, pRS511, pRS513, pRS514, pRS265, pRS279, pRS281, and pRS282.

Table S1. Summary of the generated DNA constructs.

ID	Name	Encoded protein	Aplication
pRS289	PNPaseWT-FLAG pcDNA5FRTTO	Fusion of PNPase with FLAG tag	Generation of stable 293 cell line
pRS292	PNPase_R446E-FLAG pcDNA5FRTTO	FLAG tag fusion of PNPase carrying point mutation R446E	Generation of stable 293 cell line
pRS448	PNPase_si1Res-FLAG pcDNA5FRTTO	FLAG tag fusion of PNPase carrying silent mutations in region complementary to siRNA siPN1	Generation of stable 293 cell line
pRS449	PNPase_ si2Res-FLAG pcDNA5FRTTO	FLAG tag fusion of PNPase carrying silent mutations in region complementary to siRNA siPN2	Generation of stable 293 cell line
pRS501	PNPase_R446E_ si1Res-FLAG pcDNA5FRTTO	FLAG tag fusion of PNPase carrying point mutation R446E and silent mutations in region complementary to siRNA siPN1	Generation of stable 293 cell line
pRS502	PNPase_R446E_ si2Res-FLAG pcDNA5FRTTO	FLAG tag fusion of PNPase carrying point mutation R446E and silent mutations in region complementary to siRNA siPN2	Generation of stable 293 cell line
pRS510	PNPase_si1Res-EGFP pcDNA5FRTTO	EGFP fusion of PNPase carrying silent mutations in region complementary to siRNA siPN1	Generation of stable 293 cell line
pRS511	PNPase_si2Res-EGFP pcDNA5FRTTO	EGFP fusion of PNPase carrying silent mutations in region complementary to siRNA siPN2	Generation of stable 293 cell line
pRS513	PNPase_R446E_ si1Res-EGFP pcDNA5FRTTO	EGFP fusion of PNPase carrying point mutation R446E and silent mutations in region complementary to siRNA siPN1	Generation of stable 293 cell line
pRS514	PNPase_R446E_ si2Res-EGFP pcDNA5FRTTO	EGFP fusion of PNPase carrying point mutation R446E and silent mutations in region complementary to siRNA siPN2	Generation of stable 293 cell line
pRS265	hSuv3WT-TAP pcDNA5FRTTO	TAP tag fusion of hSuv3	Generation of stable 293 cell line
pRS279	hSuv3_Δ510-514-TAP pcDNA5FRTTO	TAP tag fusion of hSuv3 with deletion of residues 510-514	Generation of stable 293 cell line
pRS281	hSuv3_Δ510-514- pcDNA5FRTTO	hSuv3 with deletion of residues 510-514	Generation of stable 293 cell line
pRS282	hSuv3_G207V/Δ510-514 pcDNA5FRTTO	hSuv3 carrying point mutation G207V and deletion of residues 510-514	Generation of stable 293 cell line
pRS472	hSuv3-pmCherryN1	Fusion of hSuv3 with mCherry	FLIM-FRET study
pRS473	PNP-pEGFPN1	Fusion of hSuv3 with EGFP	FLIM-FRET study
pRS407	hSuv3WT-pBiFC-VN173	Fusion of hSuv3 with N-termini of Venus	BiFC analysis
pRS408	hSuv3_Δ510-514 pBiFC-VN173	Fusion of hSuv3 carrying deletion of residues 510-514 with N-termini of Venus	BiFC analysis
pRS409	hSuv3WT-pBiFC-VC155	Fusion of hSuv3 with C-termini of Venus	BiFC analysis
pRS410	hSuv3_Δ510-514-pBiFC-VC155	Fusion of hSuv3 carrying deletion of residues 510-514 with C-termini of Venus	BiFC analysis
pRS411	PNPaseWT-pBiFC-VN173	Fusion of PNPase with N-termini of Venus	BiFC analysis
pRS412	PNPaseWT-pBiFC-VC155	Fusion of PNPase with C-termini of Venus	BiFC analysis
pRS415	hSuv3_G207V-pBiFC-VC155	Fusion of hSuv3 carrying carrying point mutation G207V with C-termini of Venus	BiFC analysis
pRS504	PNPase_R446E-pBiFC-VN173	Fusion of PNPase carrying point mutation R446E with N-termini of Venus	BiFC analysis

Western blotting

Protein samples were prepared and processed as described previously . Western analysis was performed according to standard procedures using the following antibodies: goat polyclonal anti-PNPase (N-18, sc-49315, Santa Cruz Biotechnology), anti-Hsp60 (K-19, sc-1722, Santa Cruz Biotechnology), mouse monoclonal anti-AIF (E-1, sc-13116, Santa Cruz Biotechnology) anti-FLAG (M2, F3165, Sigma), anti-ATP5A (ab14748, Abcam), anti-SDHB (ab14714 Abcam), anti-UQCRC2 (ab14745; Abcam), anti-COX2 (ab110258, Abcam), rabbit polyclonal anti-hSuv3, mouse monoclonal anti-actin (A5441, Sigma). Primary antibodies were detected using peroxidase conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit (401215, 401393, respectively, Calbiochem), rabbit anti-goat (A5420, Sigma)) and the Immun-Star™ WesternC™ Chemiluminescence Kit (Bio-Rad) according to the manufacturer's recommendations.

Northern blot analysis

Total RNA was isolated by TRI Reagent (Sigma) using the manufacturer's protocol and analyzed essentially as described previously (Szczesny et al, 2010). 3 μg of total RNA was dissolved in denaturing solution and run on a 1% agarose gel as described previously. Subsequently, RNA was transferred to Nytran-N membrane (Schleicher & Schuell BioScience), UV cross-linked and stained with methylene blue solution. For detection of transcripts, ND2, COX1, and COX2 probes were labeled with [α-^{32}P] dATP (Hartmann Analytic) using a HexaLabel DNA Labeling Kit (Fermentas), whereas strand specific hybridizations were performed with [α-^{32}P] UTP (Hartmann Analytic) labeled riboprobes prepared by transcription *in vitro* using the T7 transcription kit (Fermentas). In both cases, PCR products containing SP6 or T7 promoter sequences and corresponding to the following fragments of human mtDNA were used as templates: 904–1307 (12S rRNA), 4807–5172 (ND2), 5587–5891 (tRNA^{A,N,C,Y}), 5904–6251 (COX1), 7586–7900 (COX2), 10131–10375 (ND3), 15088-15499 (CytB). Hybridizations were performed in PerfectHyb Plus buffer (Sigma) at 65ºC. Membranes were exposed to PhosphorImager screens (FujiFilm), which were scanned following exposure by a Typhoon FLA 9000 scanner (GE Healthcare). Data were analyzed by Multi Gauge V3.0 software (FujiFilm).

Isolation of total DNA and Southern blot analysis

Total DNA was isolated by phenol-chloroform extraction. 2-3 x 10^6 of cells were harvested in RSB buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 10 mM EDTA) containing proteinase K (400 µg/ml, Fermentas) and RNase A (150 µg/ml, Fermentas) and lysed by addition of SDS to 0,9%. Then, cells were incubated for 3.5 hrs at 37°C with gentle mixing every 15-20 minutes. After the incubation DNA was purified by sequential extraction with equal volumes of phenol, phenol-chloroform and chloroform (all from Sigma). DNA was precipitated by adding 0.1 volume of 10 M ammonium acetate and 1 volume of isopropanol and mixing. Mixtures were incubated for 30 minutes at room temperature and centrifuged (16400 x g, 30 minutes, 18°C). DNA was washed with 75% ethanol and dissolved in water.

To analyze the level of mtDNA total DNA was digested with a mixture of NcoI/DraI enzymes (both from Fermentas) overnight and 2 µg were resolved by standard electrophoresis in (1%) agarose gel. Subsequently, the gel was immersed and mixed for 0.5 h in each of the following: depurination solution (0.2 M HCl), denaturation solution (1.5 M NaCl, 0.5 M NaOH) and neutralizating solution (0.5 M Tris-Cl pH 7.0, 3 M NaCl) with brief rinsing with water between solutions. DNA was transferred to Nytran-N membrane (Whatman, Schleicher & Schuell BioScience) by overnight upward capillary transfer using 10 x SSC (1.5 M sodium chloride, 150 mM sodium citrate) and immobilized by ultraviolet-crosslinking. Hybridizations were performed overnight in PerfectHyb Plus buffer (Sigma) at 64°C. Mitochondrial and nuclear DNA were detected using [α-^{32}P] dATP (Hartmann Analytic) labeled probes complementary to ND2 and 28rDNA genes, respectively. Probes were prepared by random priming with HexaLabel DNA Labeling Kit (Fermentas). Probe templates were PCR products amplified with the following primers: ND2 - RSZ266 taatacgactcactatagggtctgagtcccagaggttac and RSZ267 atttaggtgacactatagaattcaggt gcgagatagtag; 28rDNA - RSZ547 gcctagcagccgacttagaactgg and RSZ548 ggcctttcattattctacacc tc (28rDNA). After hybrydization membranes were exposed to PhosphorImager screens (FujiFilm), which were then scanned by a Typhoon FLA 9000 scanner (GE Healthcare). Data were analyzed by Multi Gauge V3.0 software (FujiFilm).

Submitochondrial fractionation

Mitochondria were isolated as described previously (Szczesny et al., 2010). Mitochondria were resuspended in HomB buffer (40mM Tris-HCl pH 7.6, 25mM NaCl, 5mM $MgCl_2$) with indicated concentrations of digitonin or lysed with 1% TritonX-100 and incubated on ice for 10 minutes. Subsequently one volume of HomB buffer was added and mitoplasts were

centrifuged for 2 minutes at 8000 x g. Supernatant was removed and pellet was resuspended in HomB buffer, which was supplemented with proteinase K [25 μg/mL] when indicated. Resuspended mitoplasts, intact mitochondria or Triton-lysed mitochondria were incubated for 45 minutes at 37ºC. After incubation 1mM PMSF was added to inhibit proteinase K. Mitoplasts or mitochondria were pelleted and lysed in HomB buffer with 1% Triton X-100, 1mM PMSF and 1x concentrated proteases inhibitors cocktail (Roche). The same volume of each lysates were analysed by western blot.