Supplementary Figures and Legends

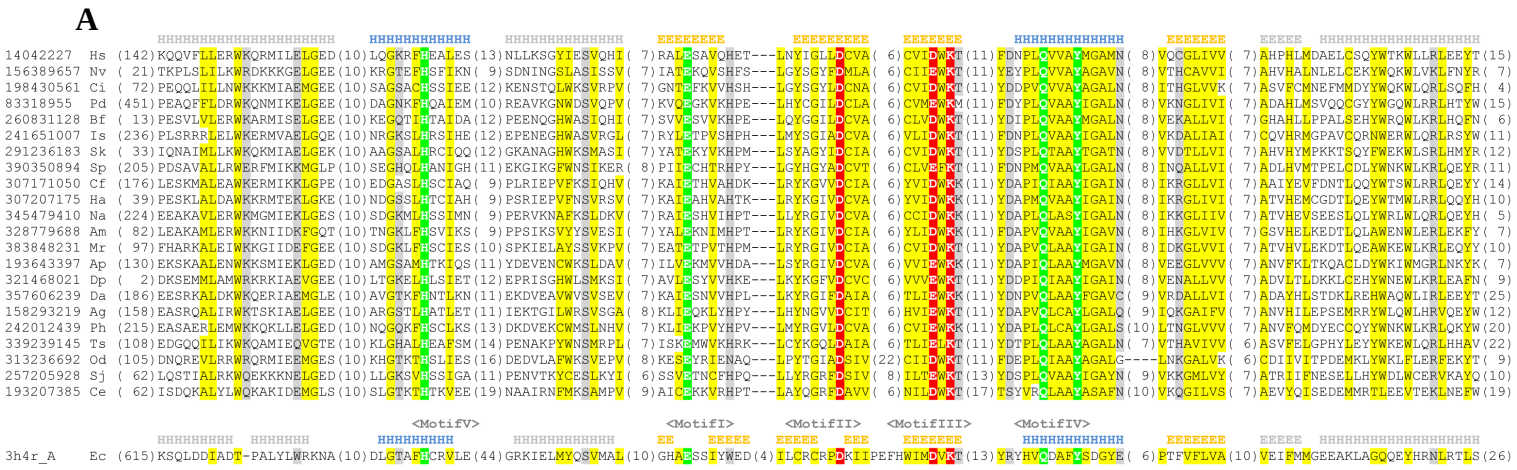


Figure S1. (A) Multiple sequence alignment for selected Ddk1-like proteins and RecE exonuclease. Residue conservation is denoted by the following scheme: uncharged, yellow; charged or polar, grey; invariant PD-(D/E)XK motif residues, red; additional conserved active site residues, green. Locations of predicted (Ddk1, gi|14042227) and observed (RecE, pdb|3h4r) secondary structure elements are marked above the corresponding sequences. Locations of sequence motifs (Motifs I-V) described for RecE are also shown. Abbreviations of the species names: *Hs, Homo sapiens; Nv, Nematostella vectensis; Ci, Ciona intestinalis; Pd, Platynereis dumerilii; Bf, Branchiostoma floridae; Is, Ixodes scapularis; Sk, Saccoglossus kowalevskii; Sp, Strongylocentrotus purpuratus; Cf, Camponotus floridanus; Ha, Harpegnathos saltator; Na, Nasonia vitripennis; Am, Apis mellifera; Mr, Megachile rotundata; Ap, Acyrthosiphon pisum; Dp, Daphnia pulex; Da, Danaus plexippus; Ag, Anopheles gambiae; Ph, Pediculus humanus corporis; Ts, Trichinella spiralis; Od, Oikopleura dioica; Sj, Schistosoma japonicum; Ce, Caenorhabditis elegans; Ec, Escherichia coli.* **(B)** CLANS clustering for the closest homologs of human Ddk1. Distinguishable taxonomic groups at arbitrary chosen levels are shown in separate colors. Dark grey and light grey lines represent BLAST pairwise scores below 1E-40 and above 1E-40, respectively. Colors were chosen from Brewer palette (qualitative set 1).

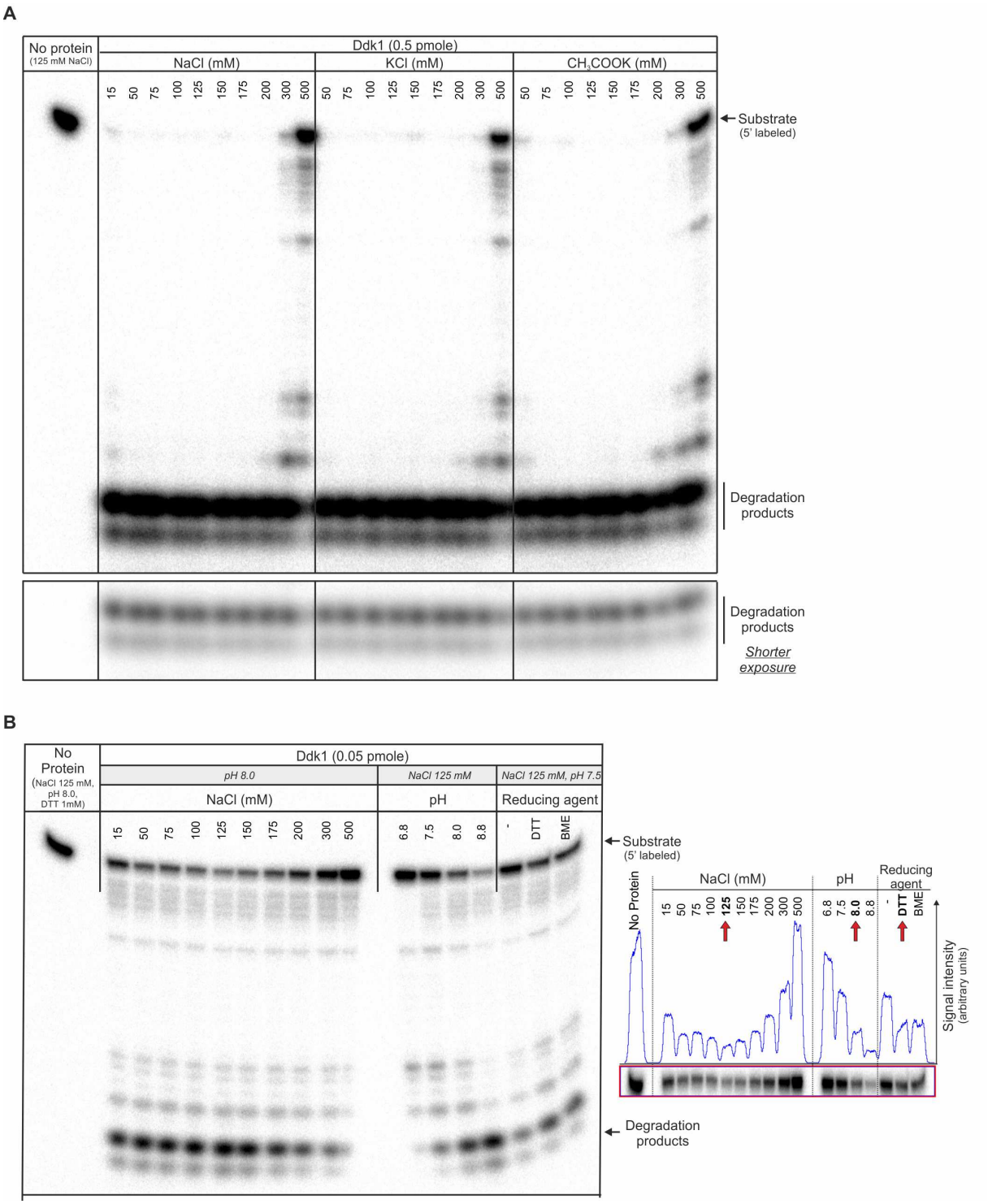


Figure S2. Determination of optimum conditions for Ddk1 *in vitro* activity. (A) 0.5 pmole of Ddk1 was incubated with 10 pmoles of 5´ labeled v81 substrate for 20 minutes at 37 °C in buffer containing BSA (0.1 μg/μl), $MgCl_2$ (5.12 mM), Tris-HCl pH 8.0 (10 mM), DTT (1 mM) and indicated amounts of salt. (B) 0.05 pmole of Ddk1 was incubated with 10 pmoles of 5´ labeled v81 substrate for 20 minutes at 37 °C in a buffer containing BSA (0.1 μg/μl), $MgCl_2$ (5.12 mM), NaCl (indicated concentration), 10 mM Tris-HCl (indicated pH). DTT or BME denote that the reaction mixture contained 1 mM dithiothreitol or beta-mercaptoethanol, respectively. The graph on the right presents the substrate signal intensity profile. Red arrows indicate components of standard Ddk1 reaction buffer.

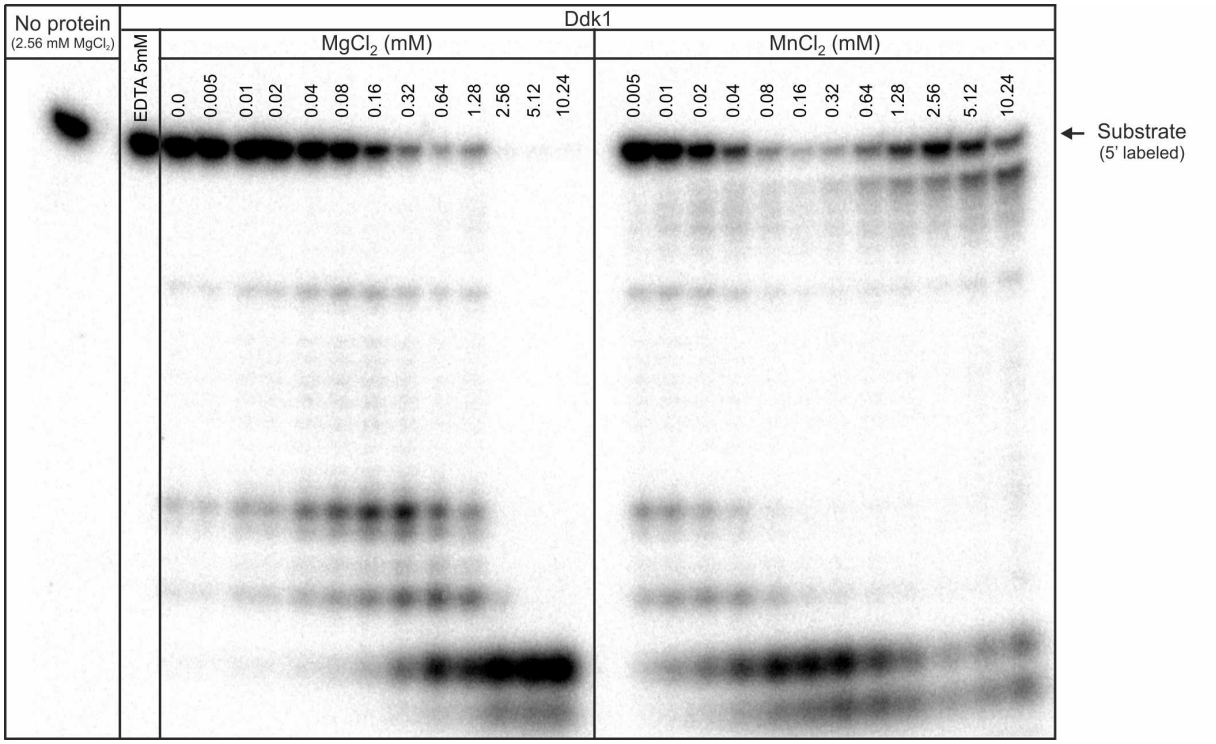


Figure S3. Ddk1 requires divalent metal ions for the activity. Ddk1 (0.5 pmole) was incubated with 10 pmoles of 5´ labeled v81 substrate for 20 minutes at 37 °C in a buffer containing BSA (0.1 μg/μl), Tris-HCl pH 8.0 (10 mM), DTT (1 mM) and the indicated amounts of $MgCl_2$ or $MnCl_2$. The presence of EDTA (5 mM) instead of Mg^{2+} or Mn^{2+} completely abolished the activity of Ddk1.

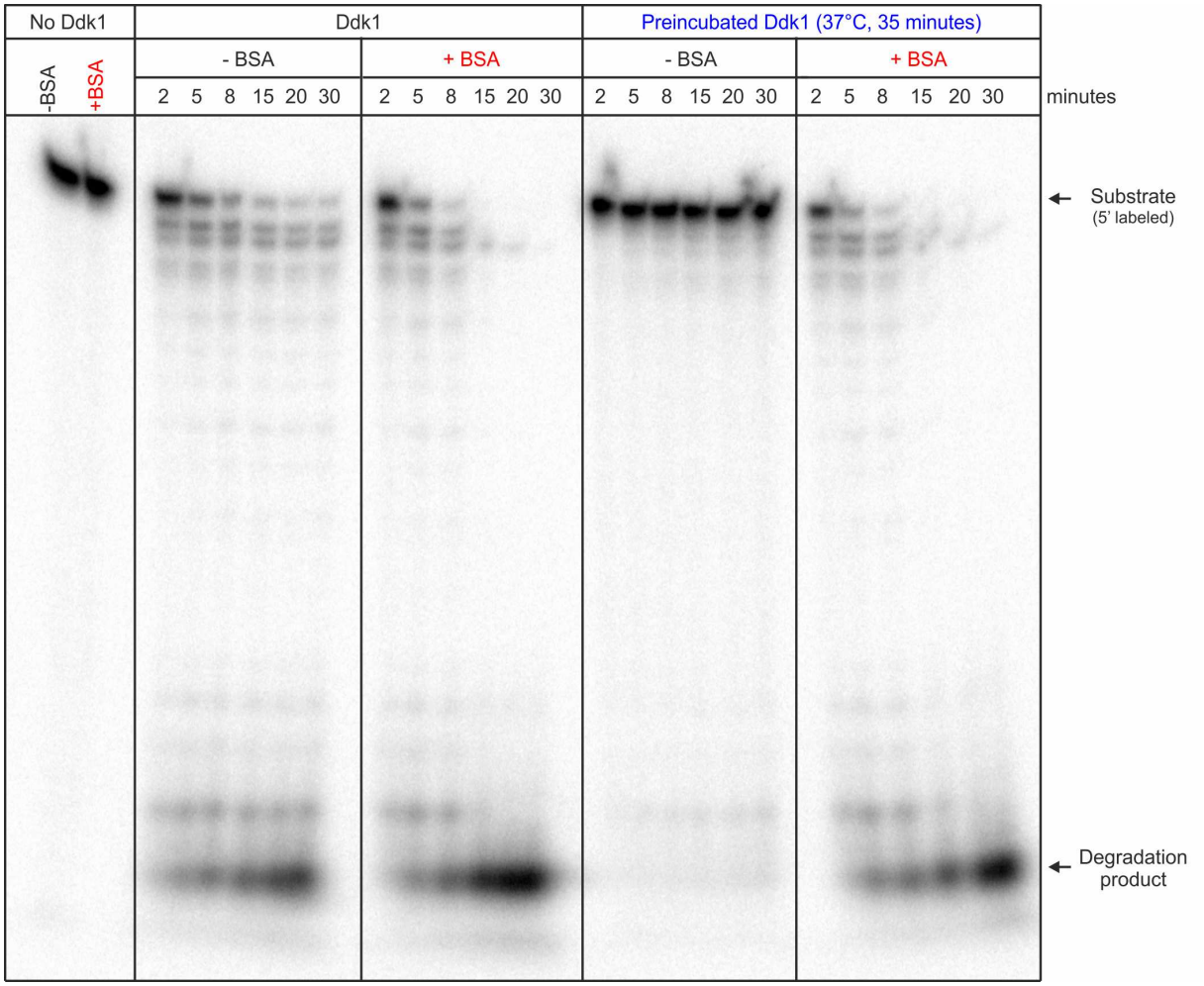


Figure S4. BSA stabilizes the Ddk1 activity. Ddk1 (0.5 pmole) was preincubated in a reaction buffer ($MgCl_2$ 5.12 mM, Tris-HCl pH 8.0 10 mM, DTT 1 mM, NaCl 125 mM) supplemented (or not) with BSA (0.1 µg/µl) for 35 minutes at 37 °C. Afterwards, 44DNA substrate (10 pmoles) was added and reactions were carried out for the indicated time at 37°C. Analogous reactions were performed using untreated Ddk1. Both reactions sets were done at the same time using Ddk1 from the same aliquot.

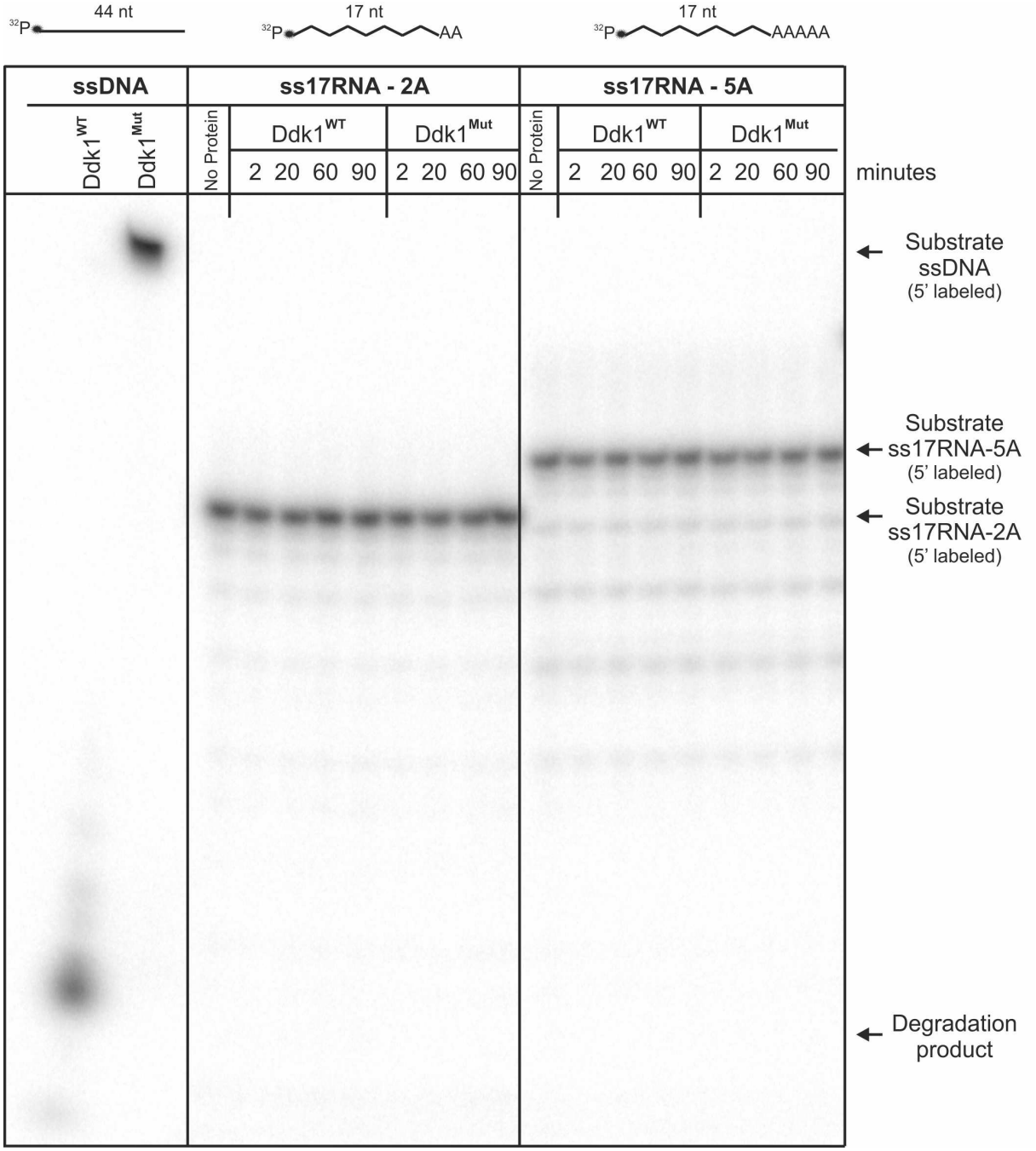


Figure S5. Ddk1 does not degrade 17 nt RNA with short A tail. Wild-type or inactive (Mut, D251N K253A) Ddk1 (0.5 pmole) was incubated with 10 pmoles of the denoted substrates (44DNA, RNA17-2A, RNA17-5A) under standard conditions for the indicated time at 37 °C. Products were analyzed by 15% urea-PAGE.

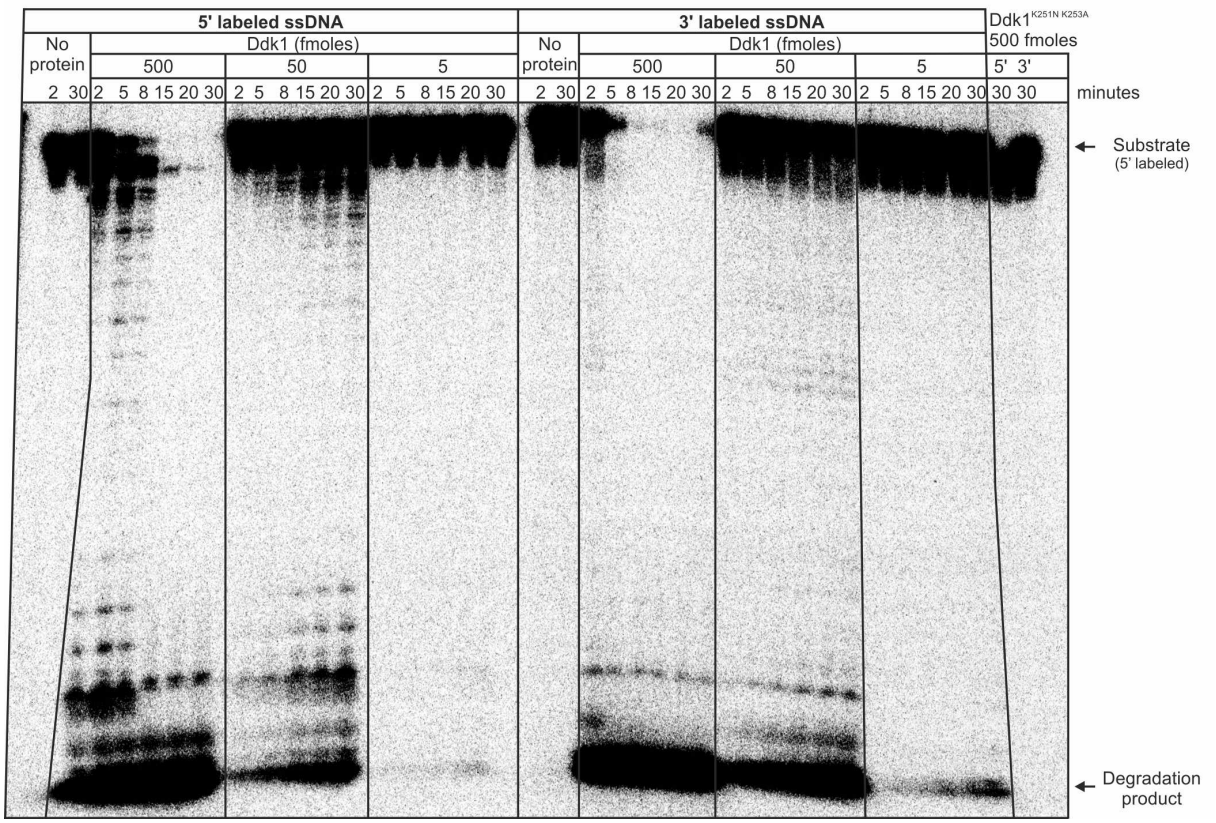


Figure S6. Ddk1 can act in both directions. Longer exposure of Figure 5 from the main text. Ddk1 activity was assayed using 5′ or 3′ labeled single-stranded 44DNA substrate. Reactions were performed in standard conditions using the indicated amounts of enzyme for the indicated time. Products were resolved using 20% urea-PAGE.

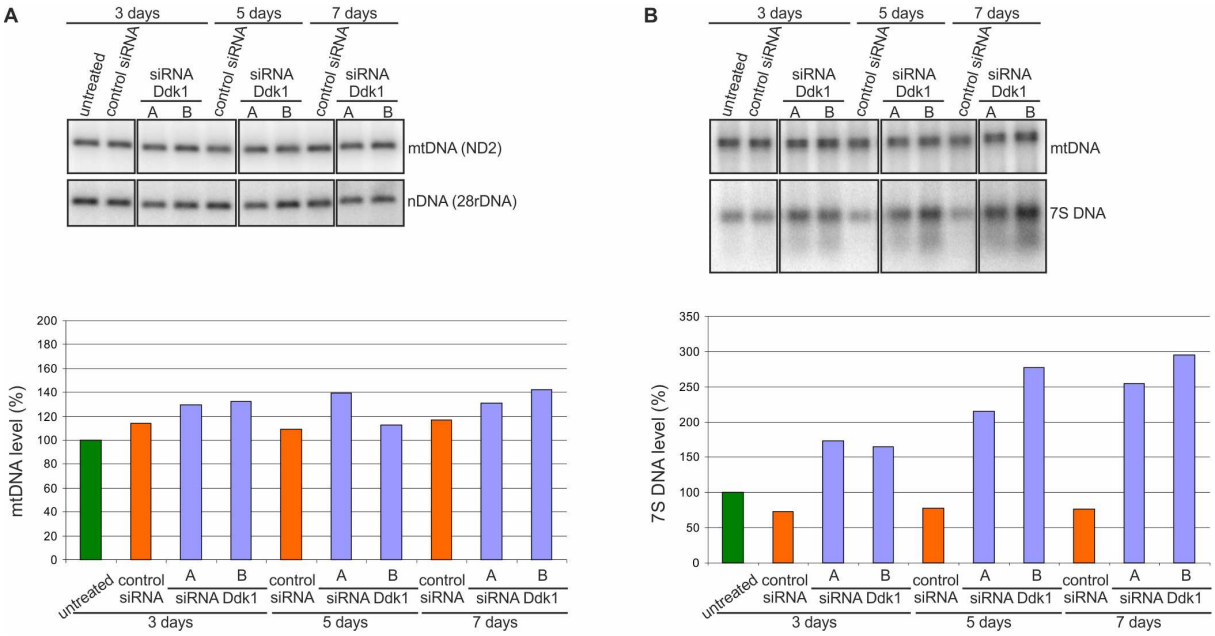


Figure S7. Influence of prolonged Ddk1 silencing on the level of mtDNA and 7S DNA. HeLa cells were transfected with control siRNA or two different Ddk1-specific siRNAs and collected after 3 days or subjected to a second transfection and collected after an additional 2 or 4 days. Experiments were performed in two technical repeats (two sets of cultures transfected at the same time) and an equal number of cells from different repeats was mixed after collection. The level of mtDNA (A) and 7S DNA (B) was examined using Southern blots. Graphs below autoradiograms present the calculated levels. The signal arising from hybridization to nuclear 28rDNA gene (nDNA) (A) or mitochondrial genome (mtDNA) (B) was used as a loading control for quantification.

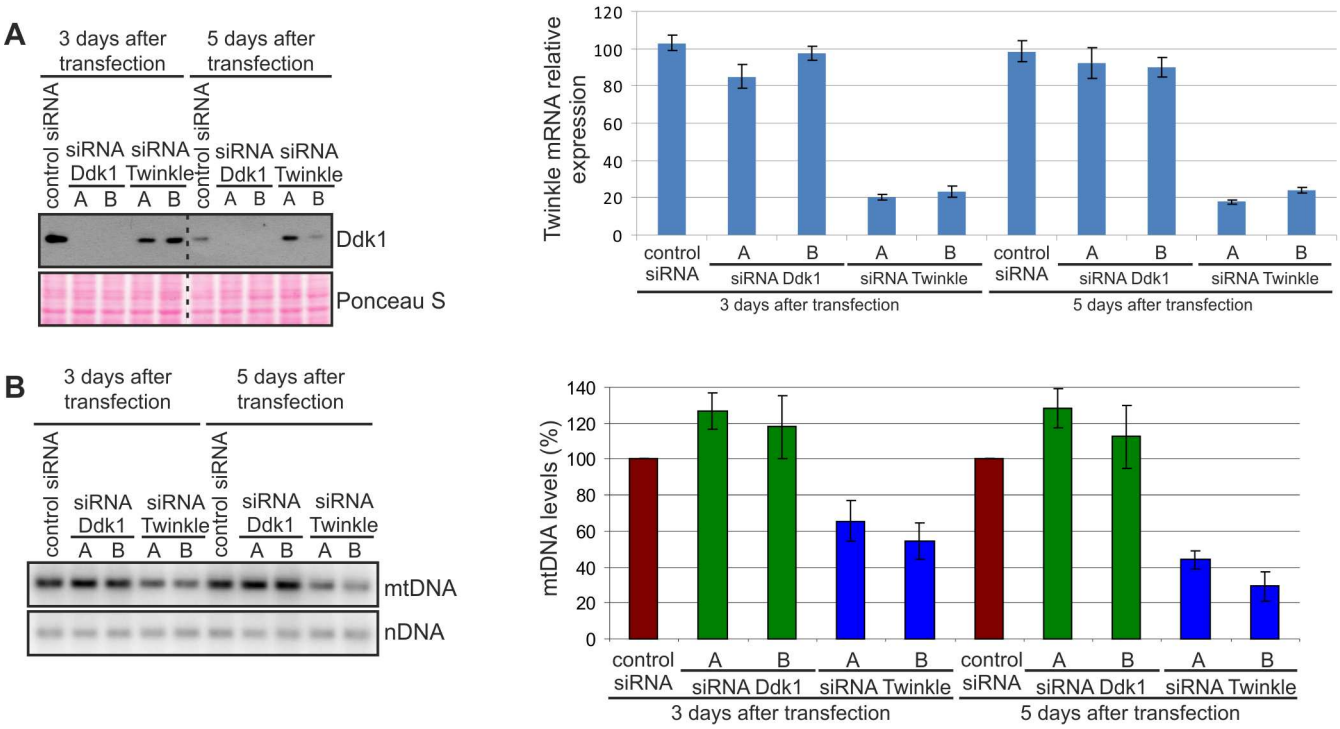


Figure S8. Silencing of Twinkle results in depletion of mtDNA. HeLa cells were transfected with control siRNA, two different Ddk1-specific siRNAs or two different Twinkle-specific siRNAs and collected 3 days or 5 days after transfection. (A) Western blot analysis of Ddk1 level. Ponceau S staining of the membrane was performed for standardization. Levels of Twinkle mRNA measured using qPCR (graph, right). (B) The level of mtDNA was examined using Southern blots. Graph present the mean values obtained in three independent experiments. Error bars represent standard deviation. The signal arising from hybridization to the nuclear 28rDNA gene (nDNA) was used as a loading control.

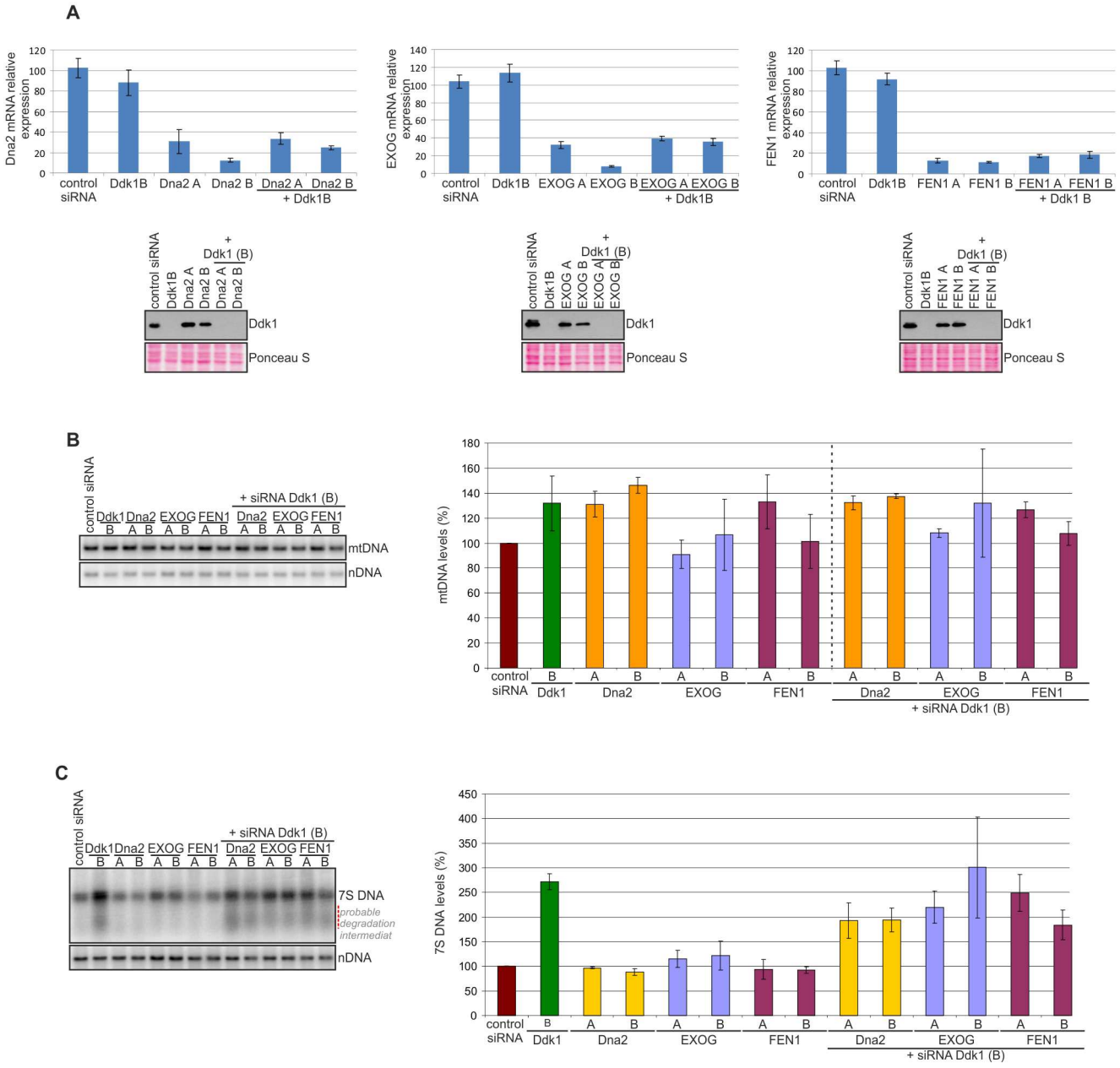


Figure S9. Influence of silencing of mitochondrial DNases on the level of mtDNA and 7S DNA. HeLa cells were transfected with indicated siRNAs and collected 3 days after transfection. (A) Graphs show levels of investigated mRNAs measured using qPCR. Western blot analysis of Ddk1 level is presented below graph. Ponceau S staining of the membranes was performed for standardization. (B, C) The level of mtDNA (B) and 7S DNA (C) was examined using Southern blots. Graphs present the mean values obtained in two independent experiments. Error bars represent standard deviation. The signal arising from hybridization to the nuclear 28rDNA gene (nDNA) was used as a loading control. (C) Dashed red line indicates probable degradation intermediates.

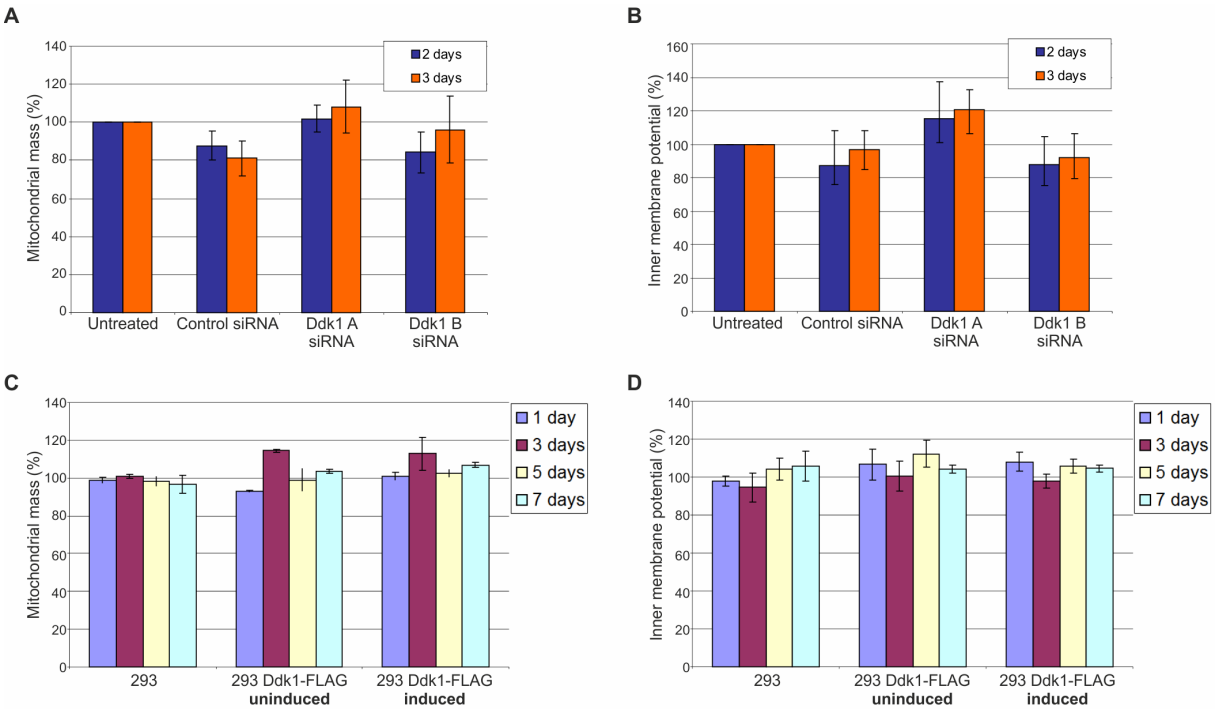
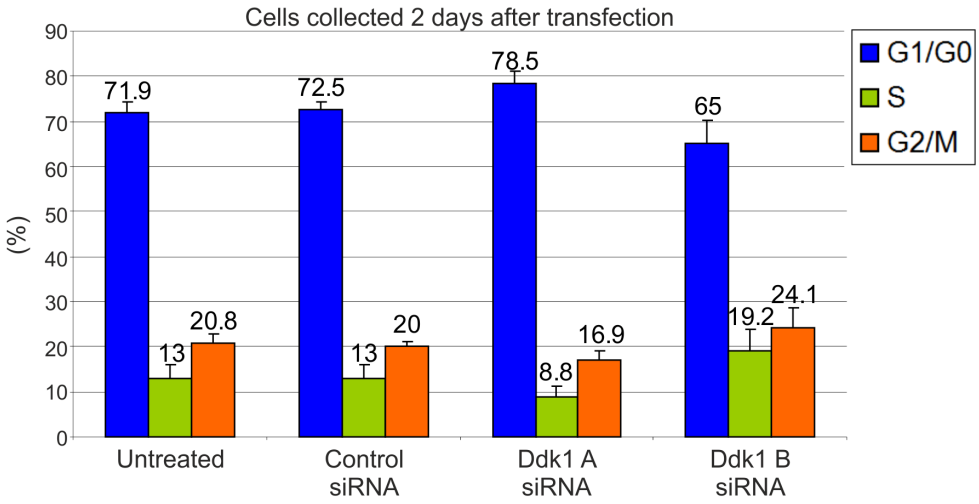


Figure S10. Neither silencing nor overexpression of Ddk1 affects mitochondrial inner membrane potential or mitochondrial mass. (A, B) HeLa cells were transfected with indicated siRNA or untreated and collected after 2 or 3 days. After collection, cells were stained with MitoTracker Red CMXRos or MitoTracker Green and analyzed by flow cytometry to measure mitochondrial inner membrane potential (A) or mitochondrial mass (B), respectively. Graphs present the mean values obtained in three independent experiments. Error bars represent standard deviation. The signal arising from untreated cells in a given set of samples was regarded as 100% for calculation purposes. (C, D) Parental 293 cells or their Ddk1-FLAG derivatives were induced (or not) and collected after the indicated time. Cells were stained with MitoTracker Red CMXRos or MitoTracker Green and analyzed by flow cytometry to measure mitochondrial inner membrane potential (C) or mitochondrial mass (D), respectively. Graphs present the mean values obtained in three independent experiments. Error bars represent standard deviation. The lowest signal registered in a sample set was regarded as 100% for calculation purposes.

A



B

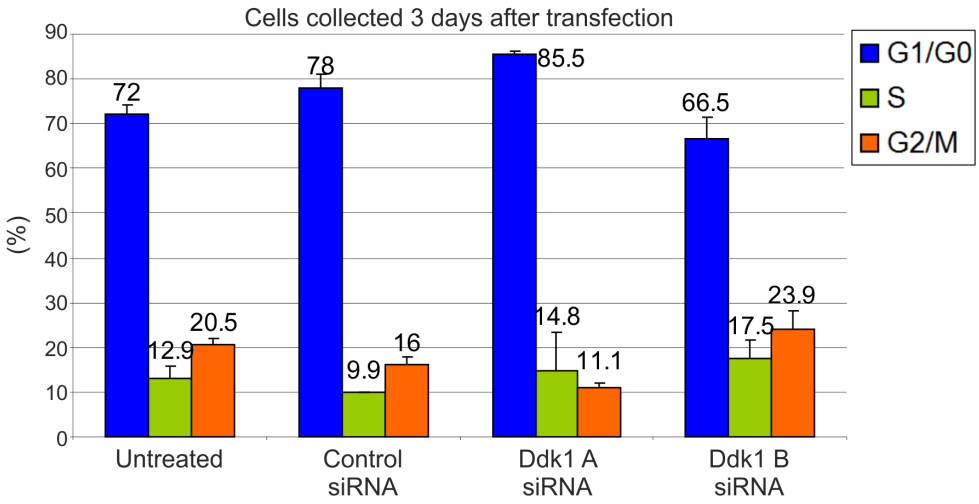


Figure S11. Depletion of Ddk1 has no considerable effect on the cell cycle profile. HeLa cells were transfected with control siRNA or two different Ddk1-specific siRNAs and collected after 2 (A) or 3 (B) days. Cells were analyzed by flow cytometry after propidium iodide staining. Graphs present the mean values obtained in three independent experiments. Error bars represent standard deviation. A manual gating was performed using the Flowing Software. Doublets were removed based on FL2-A and FL-2W analysis. Slight changes in cell cycle distribution were observed. However, the effect was different for the two siRNAs used to silence Ddk1. Two days after transfection with siRNADdk1A we observed a 6% increase in the level of G1/G0 cells and a slight decrease in the S and G2/M phases (upper graph). In contrast, transfection with siRNADdk1B resulted in a decrease in the number of the G1/G0 cells (7%) and concomitant accumulation of cells in the S and G2/M phases (upper graph). Similar, inconsistent changes were observed three days after siRNA transfection (lower graph). This analysis shows that changes in the cell cycle profile were too small to account for the observed 2-3 fold of increase in the 7S DNA levels (Figure 7C, main text). In addition, the inconsistencies in the influence of Ddk1-specific siRNAs on the cell cycle strongly indicate that alterations in the cell cycle profile arise from non-specific siRNA effects rather than Ddk1 depletion. (A) Differences between cells transfected with control siRNA and other samples are not statistically significant, apart from G0/G1 fraction from sample treated with siRNADdk1A (P=0.0383, the Student's t-test). (B) Differences between cells transfected with control siRNA and other samples are statistically significant (P values range from 0.0106 to 0.0454, the Student's t-test), apart from fraction S from samples siRNADdk1A and untreated.

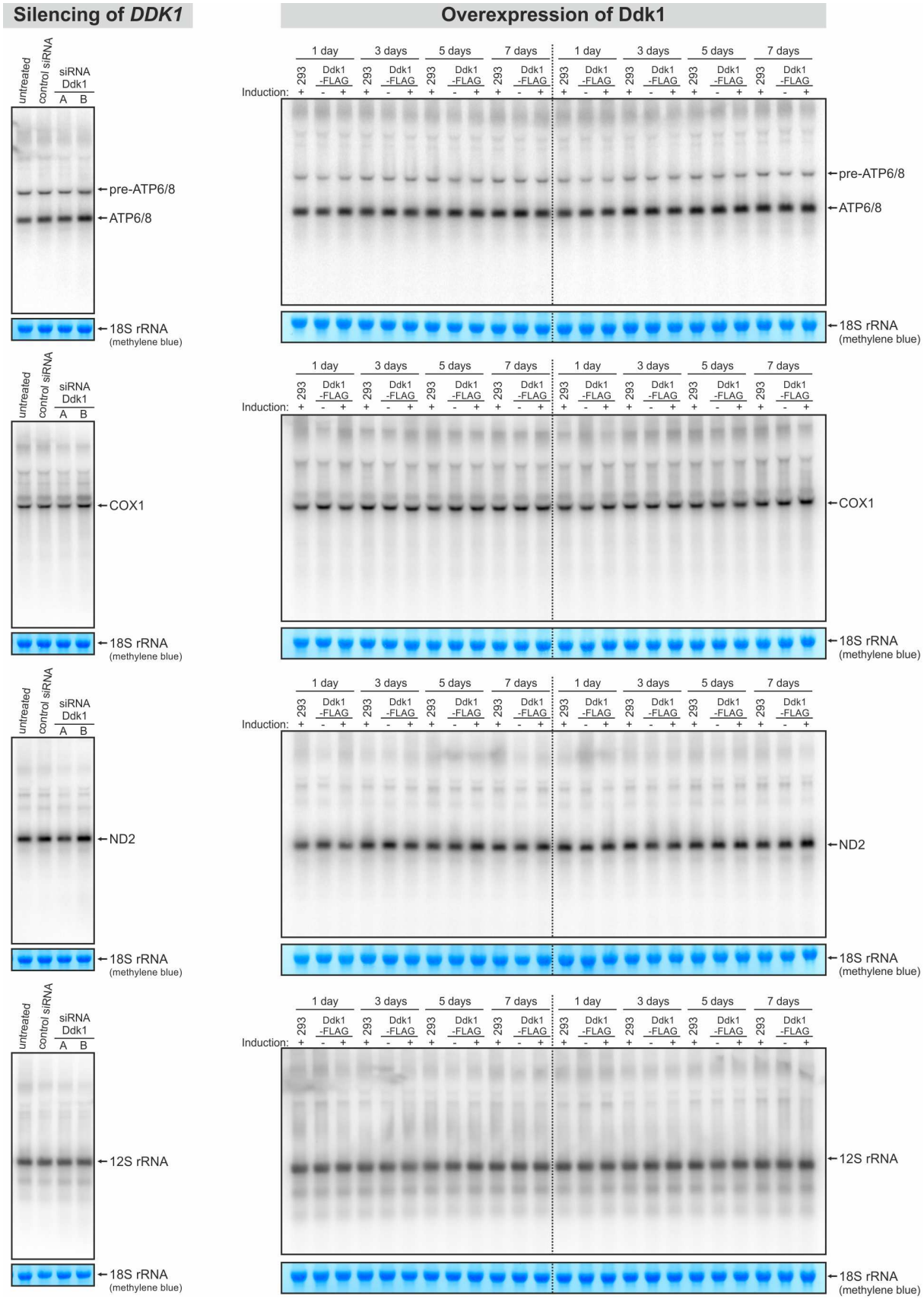


Figure S12. Neither silencing nor overexpression of Ddk1 affects RNA. RNA isolated from cells transfected with siRNA (left panels) or overexpressing Ddk1-FLAG (right panels) was analyzed by northern blotting. Probes specific for the indicated genes (both strands) were used. Cytosolic 18S rRNA staining by methylene blue is shown as a loading control. The results for two independent sample sets are shown in the case of Ddk1-FLAG overexpressing cells.

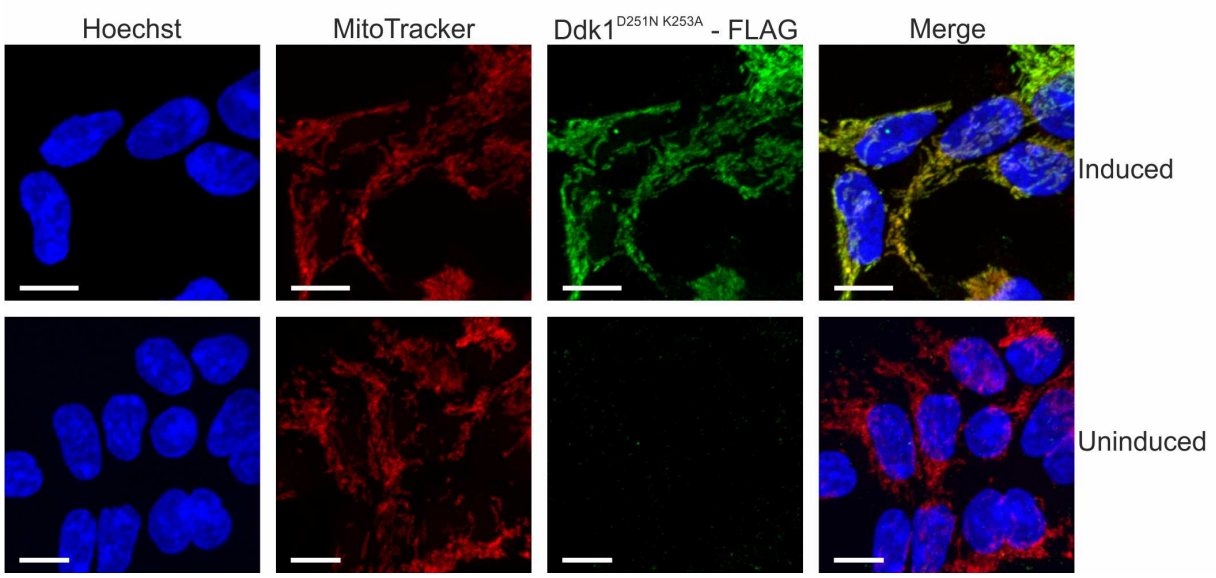


Figure S13. Subcellular localization of mutated Ddk1-FLAG in a stable inducible 293 cell line. Exogenous gene expression was induced for 24 h and cells were subjected to immunofluorescence staining and confocal microscopy. The same microscope settings were used for uninduced and induced cells. Mitochondria were labeled with MitoTracker, and Ddk1$^{D251N, K253A}$-FLAG with primary mouse anti-FLAG antibodies followed by detection with secondary AlexaFluor 488 conjugated antibodies. Nuclei were stained with Hoechst dye. The bar represents 10 μm.

Supplementary Methods

Plasmid construction

The Ddk1 coding sequence was amplified using cDNA synthesized on total RNA isolated from HeLa cells and cloned into vectors by the ligation-independent method (1). Constructs were created using pET28-HisSUMO (bacterial expression) and EGFP- or FLAG-encoding derivatives of the pcDNA5FRT/TO vector (Invitrogen, mammalian expression) modified to facilitate ligation-independent cloning (Szczesny et al., manuscript in preparation). To delete the N-terminal fragment of Ddk1, the entire pRS578 construct was amplified by PCR (primers RSZ510, RSZ511), without the fragment subjected to deletion, and the PCR product was treated with DpnI (Fermentas) and self-ligated. Substitution of catalytic residues was obtained by site-directed mutagenesis of plasmids pRS570 and pRSZ580 using oligonucleotides RSZ507 and RSZ508 and performed according to standard protocols. All constructs are listed in Table S1.

Table S1. List of generated constructs.

ID	Name	Primers used for plasmid construction	Encoded protein	Application
pRS578	Ddk1 – pETHisSUMO 8.53-2	TGAAGTCTACCAGGAACAAACCGGTGGATCCATGAAGATGAAGTTATTTCAG ATCTCAGTGGTGGTGGTGGTGCTCGAGTTATTCTGAATATTCTGGTTTC	6xHisSUMO - Full length Ddk1	Protein purification after expression in *E. coli*
pRS580	Δ21 Ddk1 - pETHisSUMO	RSZ 510:ggatccaccggtttgttcctg RSZ 511:atgtcagctgcccttgtggctttc	6xHisSUMO - Δ21 Ddk1	Protein purification after expression in *E. coli*
pRS582	Δ21 Ddk1 - D251N, K253A pETHisSUMO	RSZ507:cagggcaagctctgtgtgattaattgggcgacatcagagaaac RSZ508:gtttctctgatgtcgcccaattaatcacacagagcttgccctg	6xHisSUMO - Mutated (D251N, K253A) Δ21 Ddk1	Protein purification after expression in *E. coli*
pRS570	Ddk1-FLAG (C20orf72-TEV-FLAG, 8.56-6A	GGATCCGAAAACCTGTACTTCCAAGGAACCGGTATGAAGATGAAGTTATTTCAG GATATCACCCTGAAAATACAAATTCTCGCTAGCTTCTGAATATTCTGGTTTCTG	Full length Ddk1 - FLAG	Generation of stable 293 cell line
pRS571	Ddk1 D251N K253A-FLAG	RSZ507:cagggcaagctctgtgtgattaattgggcgacatcagagaaac RSZ508:gtttctctgatgtcgcccaattaatcacacagagcttgccctg	Mutated (D251N K253A) full length Ddk1D251N, K253A - FLAG	Generation of stable 293 cell line
pRS572	Ddk1-EGFP (C20orf72-TEV-EGFP, 8.55-5)	GGATCCGAAAACCTGTACTTCCAAGGAACCGGTATGAAGATGAAGTTATTTCAG GATATCACCCTGAAAATACAAATTCTCGCTAGCTTCTGAATATTCTGGTTTCTG	Full length Ddk1 - EGFP	Generation of stable 293 cell line

Ddk1 purification and multiangle light scattering analysis

Wild type or mutated Δ21 Ddk1 proteins were overproduced as N-terminal HIS-SUMO fusion proteins in *E. coli* cultured in an auto-induction medium. After 48 hours of culture the bacteria were pelleted, lysed by sonication, and protein extracts were subjected to purification. Recombinant protein was purified by Ni affinity chromatography, followed by SUMO protease cleavage, desalting, a second round of Ni affinity chromatography with collection of unbound material, and gel filtration. The recombinant protein has additional serine at the N-terminus left over after cleaving off the tag. All steps were done automatically using an ÄKTA express apparatus. Protein concentration was measured using NanoDrop 2000 at 280 nm. MALS-SEC (multiangle light scattering analysis combined with size exclusion chromatography) analysis was performed using the Wyatt Dawn Heleos II apparatus connected to Optilab supported with refractometer. Results were analyzed using Astra 6 software according to manufacturer's instructions.

Reverse transcription and quantitative PCR

Total RNA was isolated using TRI Reagent (Sigma) as recommended in the manufacturer's protocol. Prior to conversion to cDNA 15 µg of total RNA was treated with TURBO DNase (AM2238; Ambion) according to manufacturer's instructions and extracted with phenol:chloroform. 3 µg of total RNA was reverse transcribed using 300 ng of random primers (48190-011; Invitrogen) and SuperScript III Reverse Transcriptase (18080-044; Invitrogen) according to manufacturer's recommendations. Quantitative PCR was performed in triplicate using 1 µl of cDNA (diluted 1 to 5) and Platinum SYBR Green qPCR SuperMix-UDG (11733-046; Invitrogen). The LightCycler® 480 Real-Time PCR System (Roche) was used. Following primers were used: EXOG (RSZ704/705) – RSZ704: GCTCAGTATCTACCGAACCACT, RSZ705: AAACACCAGTCCTGACAACTTC; FEN1 (RSZ708/709) – RSZ708: CACCTGATGGGCATGTTCTAC, RSZ709: CTCGCCTGACTTGAGCTGT; DNA2 (RSZ714/715) – RSZ714: GGTGCCATACCTGTCACAAAT, RSZ715: AGGACCGACAAGTTTCTGTCTA; Twinkle (RSZ718/719) – RSZ718: GCCATGTGACACTGGTCATT, RSZ719: AACATTGTCTGCTTCCTGGC. The level of investigated transcripts was standarized to the level of GAPDH transcript, which was amplified using primers: AAGGTGAAGGTCGGAGTCAAC; GGGGTCATTGATGGCAACAATA (PrimerBank ID 8364189061 (2).

Northern blot analysis

Total RNA was isolated using TRI Reagent (Sigma) as recommended in the manufacturer's protocol and analyzed by northern blotting as described previously (3). All hybridizations were performed overnight in PerfectHyb Plus buffer (Sigma) at 64°C. PCR products containing SP6 or T7 promoter sequences (one on

each end) of the following mtDNA fragments: 904-1307 (12S rRNA), 4807-5172 (ND2), 5904-6252 (COX1) and 8631-8932 (ATP6/8) were used as templates for preparing probes labeled with [α-^{32}P] dATP (Hartmann Analytic) using the HexaLabel DNA Labeling Kit (Fermentas). Autoradiograms were obtained as for Southern blot analysis. As a loading control, the filters were incubated with methylene blue to stain rRNA.

Flow cytometry

Cell cycle analysis was performed as previously described (4). Briefly, cells were washed once with PBS and fixed with incubation in ice-cold 70% ethanol for one hour at 4 ºC. After centrifugation, cells were harvested in PBS solution containing RNase A (50 μg/ml, Sigma) and propidium iodide (50 μg/ml, Sigma) and incubated for 30 minutes at room temperature prior to flow cytometry analysis. For measurement of mitochondrial mass or mitochondrial inner membrane potential, cells (4 x 10^5) were centrifuged (300 x g, 3 minutes, room temperature) and harvested in complete medium containing MitoTracker Green FM (50 nM) or MitoTracker Red CMXRos (50 nM), respectively. After incubation at 37°C for 20 minutes, cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences), which was used for all flow cytometry.

References

1. Li, M.Z. and Elledge, S.J. (2007) Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat Methods*, **4**, 251-256.
2. Wang, X., Spandidos, A., Wang, H. and Seed, B. (2012) PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Res*, **40**, D1144-1149.
3. Szczesny, R.J., Borowski, L.S., Brzezniak, L.K., Dmochowska, A., Gewartowski, K., Bartnik, E. and Stepien, P.P. (2010) Human mitochondrial RNA turnover caught in flagranti: involvement of hSuv3p helicase in RNA surveillance. *Nucleic Acids Res*, **38**, 279-298.
4. Szczesny, R.J., Obriot, H., Paczkowska, A., Jedrzejczak, R., Dmochowska, A., Bartnik, E., Formstecher, P., Polakowska, R. and Stepien, P.P. (2007) Down-regulation of human RNA/DNA helicase SUV3 induces apoptosis by a caspase- and AIF-dependent pathway. *Biol Cell*, **99**, 323-332.