SUPPLEMENTARY FIGURES
Figure S1

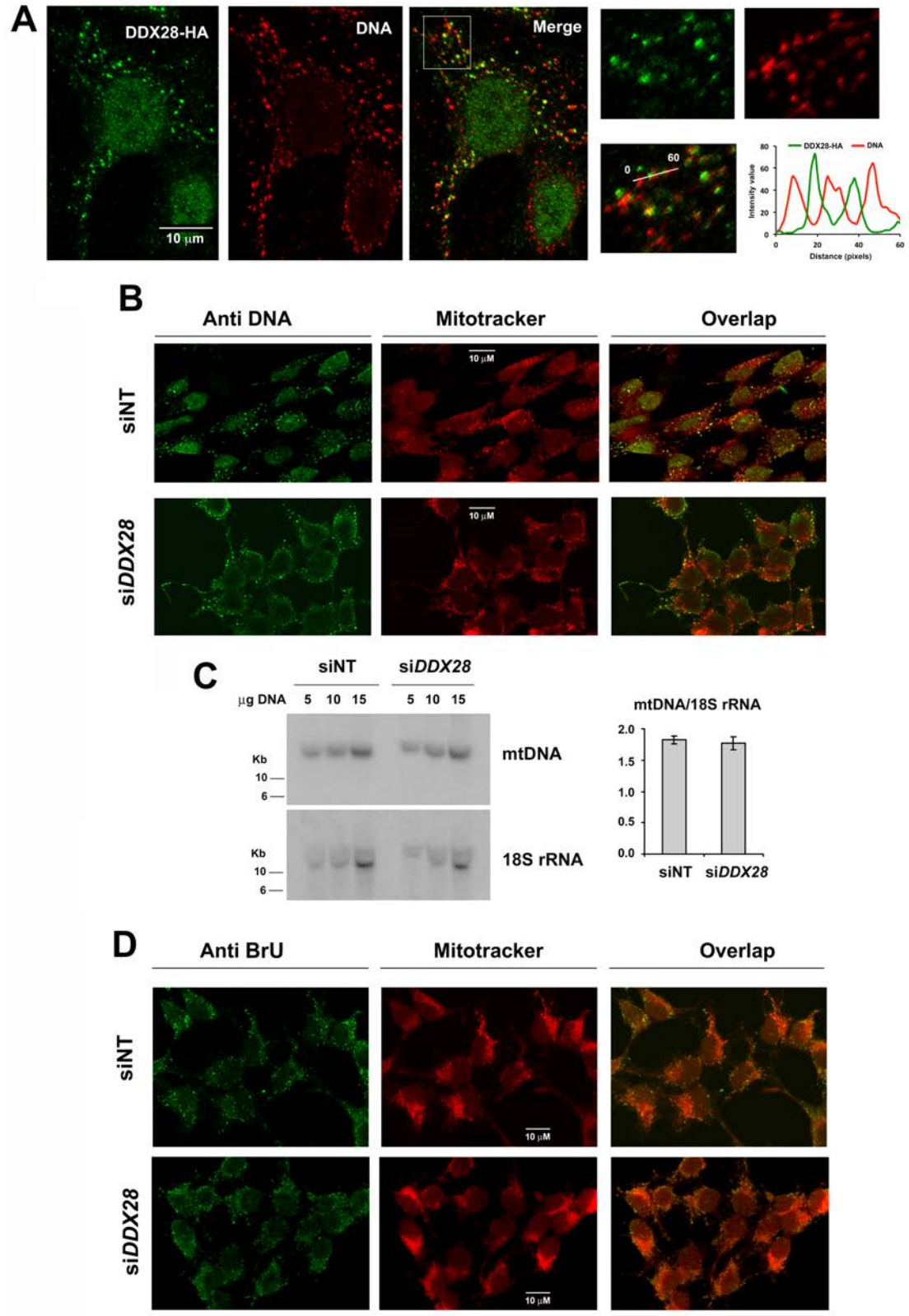
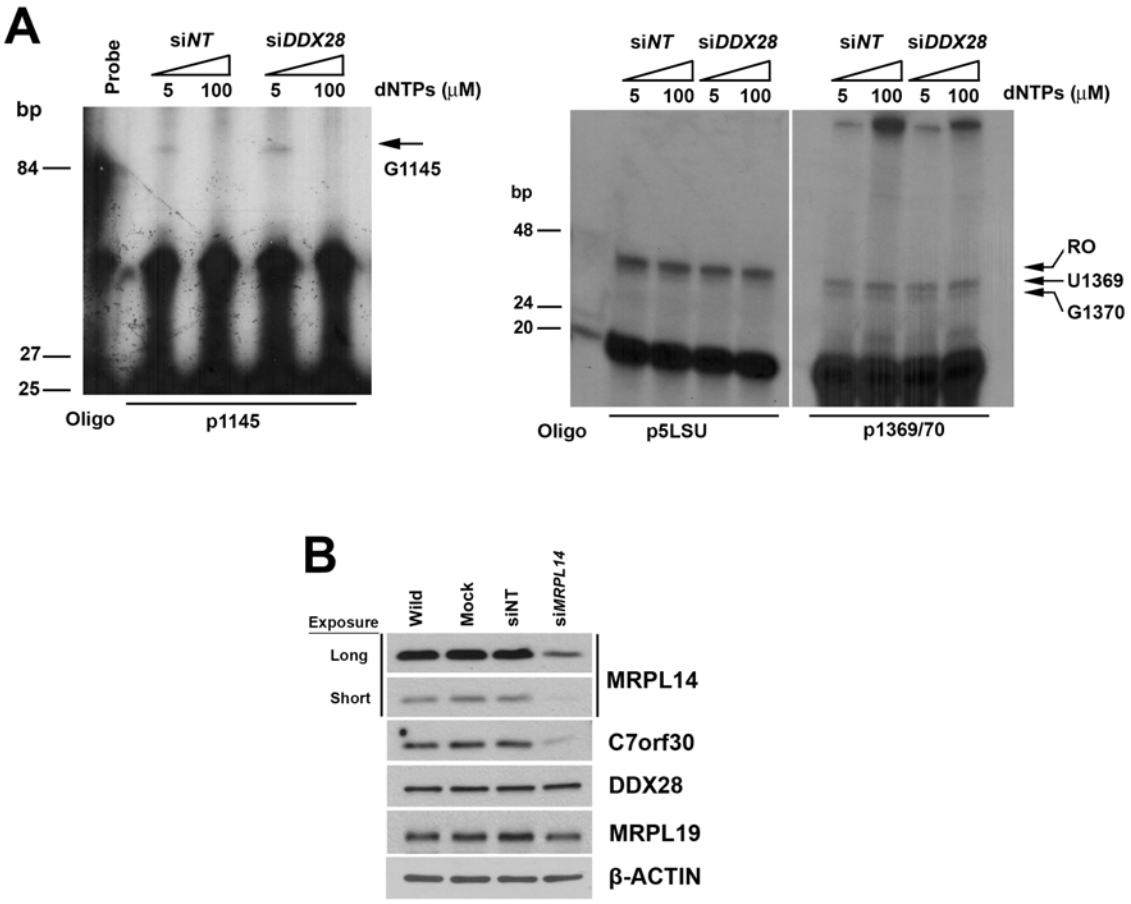


Figure S2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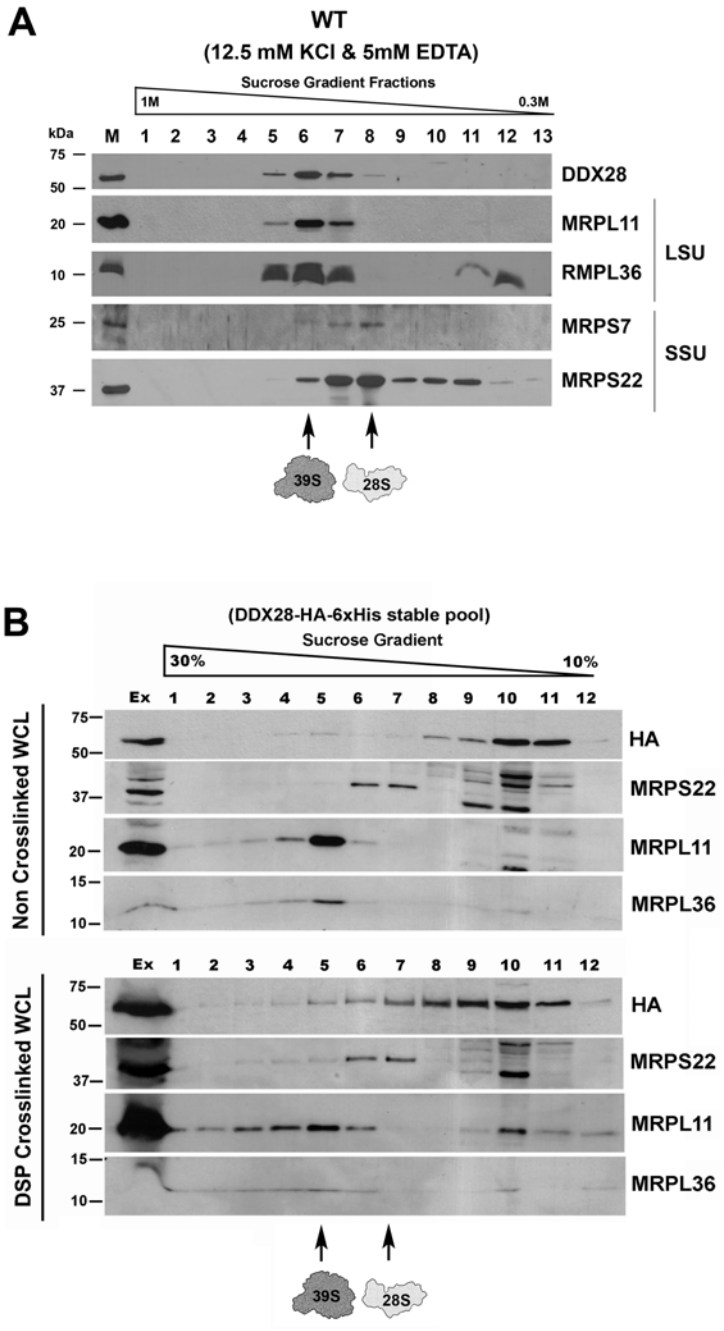
25
G1145
Oligo
p1145
siNT siDDX28 siNT siDDX28
5 100 5 100 5 100 5 100
dNTPs (μM)
bp
48
24
20
RO
U1369
G1370
Oligo
p5LSU
p1369/70
B
Wild
Mock
siNT
siMRPL14
Exposure
Long
Short
MRPL14
C7orf30
DDX28
MRPL19
β-ACTIN

Figure S3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Sucrose Gradient Fractions
1M
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kDa
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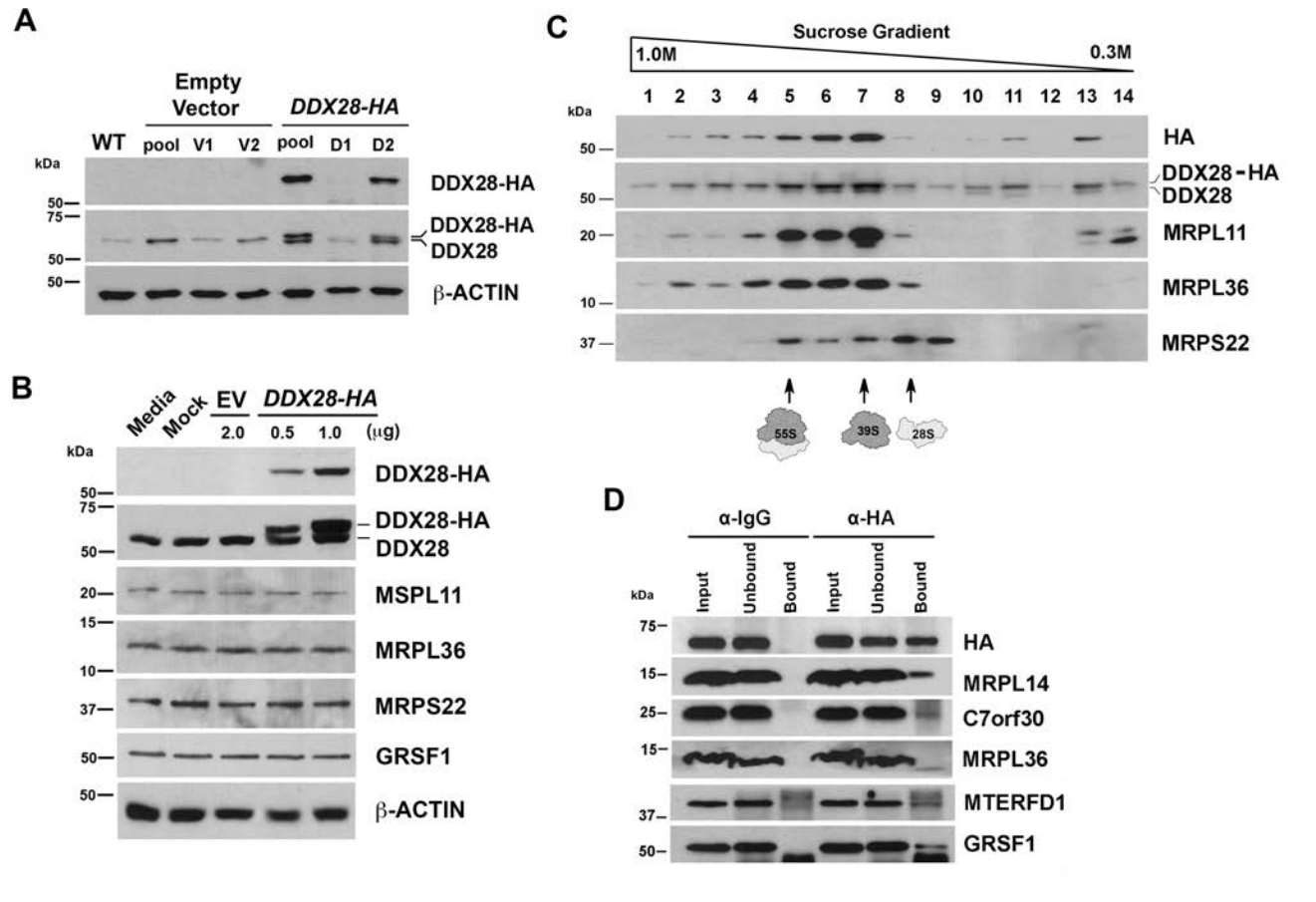
75
50
20
10
25
37
DDX28
MRPL11
RMPL36
MRPS7
MRPS22
LSU
SSU
39S
28S
B
(DDX28-HA-6xHis stable pool)
Sucrose Gradient
30%
10%
Ex 1 2 3 4 5 6 7 8 9 10 11 12
Non Crosslinked WCL
DSP Crosslinked WCL
75
50
37
20
15
10
HA
MRPS22
MRPL11
MRPL36
39S
28S

Figure S4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V1
V2
pool
D1
D2
kDa
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75
50
50
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DDX28-HA
DDX28
β-ACTIN
B
Media
Mock
EV
DDX28-HA
2.0
0.5
1.0
(μg)
kDa
50
75
50
20
15
10
37
50
50
DDX28-HA
DDX28-HA
DDX28
MSPL11
MRPL36
MRPS22
GRSF1
β-ACTIN
C
Sucrose Gradient
1.0M
0.3M
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kDa
50
50
20
10
37
HA
DDX28-HA
DDX28
MRPL11
MRPL36
MRPS22
55S
39S
28S
D
α-IgG
α-HA
Input
Unbound
Bound
Input
Unbound
Bound
kDa
75
15
25
15
37
50
HA
MRPL14
C7orf30
MRPL36
MTERFD1
GRSF1

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. DDX28 does not colocalize with mitochondrial nucleoids and *DDX28* silencing does not disturb mitochondrial DNA stability nor the formation of RNA granules. Related to Fig. 1.
(**A**) Bromouridine-treated HEK293T cells with anti-HA and anti-bromouridine antibodies and DAPI to stain the nuclei. The square marked in the "Merge" image was magnified and the graphs represent relative intensity of the fluorescence signal along the line in the magnified panel. NT, non-transformed cell.
(**B**) Immunofluorescence analysis of HEK293T cells with anti-DNA antibody and mitotracker red to visualize the mitochondrial network.
(**C**) Southern-blot analysis of mtDNA content in siNT and si*DDX28* cells. Total DNA was digested with *Pvu*II to linearize the mtDNA, separated in a 0.8% agarose gel, transferred to a Nylon membrane and the blot probed with ^{32}P-labelled probes that recognize the mtDNA and the nuclear 18S gene used as a loading control. In the right panel, the signals were digitalized and the bands quantified using the histogram option in Abode Photoshop. The bars represent average ± SD of three repetitions.
(**D**) Immunofluorescence analysis of HEK293T siNT (non-targeting) and si*DDX28* cells with anti-bromouridine antibody and mitotracker red to visualize the mitochondrial network.

Figure S2. Normal methylation of 16S rRNA in the absence of DDX28 and normal DDX28 levels in cells depleted of MRPL14. Related to Fig. 3
(**A**) Analysis of 16S rRNA methylation sites in control and si*DDX28* HEK293T cells using primer extension analyses carried out in the presence of two different dNTP concentrations, 5 **μ**M or 100 **μ**M. Primers used were specific for G1145 or U1369-G1370. The p5LSU primer was used to standardize the loading of 16S rRNA as reported (Rorbach et al., 2014). RO indicates the run-off product resulting from extending the p5LSU primer.
(**B**) Immunoblot analyses of the steady-state levels of DDX28, ribosomal proteins and assembly factors in control and si*MRPL14* HEK293T cells. An antibody against **β**-ACTIN was used as loading control.

Figure S3. DDX28 co-sediments with the large mitoribosomal subunit. Related to Fig. 4.
(**A**) Sucrose gradient sedimentation analyses of DDX28 and mitoribosomal proteins from wild-type (WT) HEK293T mitochondrial extracts prepared in the presence of 12.5 mM KCl and 5 mM EDTA).
(**B**) Sucrose gradient sedimentation analyses of DDX28 and mitoribosomal proteins using whole-cell extracts, prepared in WCE buffer [40 mM Tris– HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 1% NP40, 1x protease inhibitor cocktail (Roche)], from wild-type (WT) HEK293T mitochondria pre-treated or not with the crosslinker DSP (Dithiobis[succinimidyl propionate]). The fractions were analyzed by immunoblotting using Abs against the indicated proteins.

Figure S4. DDX28 Interacts with Large Mitoribosome Subunit Proteins and Ribosome Assembly Factors. Related to Fig. 5.
(**A**) Selection of HEK293T clones stably expressing DDX28-HA. Immunoblot analyses of the steady-state levels of DDX28 and DDX28-HA in HEK293T cells (WT) transfected with an empty vector or a construct expressing DDX28-HA. In each case, a fraction of

the pool of transfectants and individual clones (V1, V2 and D1, D2) were analyzed. An antibody against **β**-ACTIN was used as a loading control.
(**B**) Immunoblot analyses of the steady-state levels of DDX28, DDX28-HA, ribosomal proteins and assembly factors in HEK293T cells transiently expressing DDX28-HA. An antibody against β-ACTIN was used as a loading control. The micrograms of DNA construct used in transfection are indicated. EV, empty vector.
(**C**) Sucrose gradient sedimentation analyses of DDX28-HA and mitoribosomal proteins from mitochondrial extracts prepared from HEK293T cells expressing DDX28-HA.
(**D**) Immunoblot analyses of immunoprecipitates obtained from mitochondrial extracts from HEK293T cells expressing DDX28-HA, using magnetic beads and a polyclonal antibody against the HA tag. The loaded amount of bound material is 3X.

SUPPLEMENTAL TABLES

Table S1. Proteins pulled down with anti-DDX28 antibody identified by mass spectrometry. A mock pulldown with immunoglobulin was used as a control. See attached Excel Files. Related to Fig. 7.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mitochondrial Preparation and DDX28 Localization Experiments

Mitochondria were isolated from HEK293T cells essentially as described (Fernandez-Vizarra et al., 2010). The cells were resuspended in ice-cold T-K-Mg buffer (10 mM Tris-HCl/10 mM KCl/0.15 mM MgCl2; pH 7.0) and homogenized with ten passes in a homogenizer (Kimble/Kontes, Vineland, NJ). The homogenate was brought to a final concentration of 0.25 M sucrose and a postnuclear supernatant was obtained by centrifugation of the samples twice for 3 min at 1,500 x g. Mitochondria were pelleted by centrifugation for 10 min at 8,000 x g, and washed once in the T-K-Mg-Sucrose buffer. Subcellular fractionation of HEK293T mitochondria was performed as described (Clemente et al., 2013). For submitochondrial localization experiments, mitochondria from HEK293T were ruptured by sonication. Soluble and insoluble fractions were obtained by centrifugation at 50,000 x g for 15 min at 4 °C. The membrane pellet was resuspended in 0.1 M Na_2CO_3, pH 11. After 30 min on ice, the sample was centrifuged at 50,000 x g for 20 min at 4 °C to separate the soluble extrinsic from the insoluble intrinsic membrane proteins. Purified mitochondria were also resuspended in 10mM Hepes pH 7.4 buffer with or without 0.6 M sorbitol to allow mitochondria swelling and conversion to mitoplasts. Where indicated, samples were treated with 5 µg/ml proteinase K for 1 h on ice. Mitochondria and mitoplasts were recovered by centrifugation at 50,000 x g for 15 min at 4 °C and analyzed by immunoblotting.

Antibodies

The following commercial antibodies were used in this study: Total OXPHOS Human WB Antibody Cocktail, NDUFA9, complex II subunit 70 kDa Fp, UQCRC2, UQCRFS1, COXI, COXII, COXIV, ATP5A, MRPL11, MRPL12, MRPL14, MRPL19, MRPL36, MRPS7, MRM1, Hsp70, RCC1, and β-Actin from Abcam; TFAM from Abnova; VDAC from EMD Calbiochem; HA from Invitrogen; MTERFD1 from Novus Biologicals; DNA from PROGEN Biotechnik GmbH; MRPS22, MRPS27, C7orf30 and ICT1 from Proteintech Group; α-Tubulin from Santa Cruz Biotechnology; and DDX28-Prestige,

GRSF1 and BrdU from Sigma-Aldrich.

Blue Native (BN)-PAGE
The abundance of OXPHOS complexes was analyzed by Blue Native polyacrylamide gel electrophoresis (BN-PAGE) using a linear 3-12% gradient gel. Cells were collected at 6 and 9 days of *DDX28* interference. Blue native electrophoresis of the OXPHOS complexes was performed as described (Diaz et al., 2009).

Cell Respiration and Mitochondrial Respiratory Chain Enzymatic Assays
Exponentially growing HEK293T-siNT and HEK293T-si*DDX28* cells were collected by trypsinization after 6 and 9 days of transfection, pelleted, resuspended in respiratory buffer (RB; 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mg/ml BSA, 10 mM KH_2PO_4, pH 7.4). Oxygen utilization was measured polarographically in 0.3 ml RB with a Clark oxygen electrode in a micro water-jacketed cell, magnetically stirred, at 37°C (Hansatech Instruments, Norfolk, UK). After measurement of intact cell-coupled endogenous respiration, the reaction was inhibited with KCN (700 µM). CIV or cytochrome *c* oxidase activity, CI+ CIII and NADH cytochrome *c* reductase activity were measured spectrophotometrically in frozen-thaw cells as reported (Barrientos et al., 2009). The values were normalized by the activity of the TCA cycle enzyme citrate synthase (Barrientos et al., 2009).

Pulse Labeling of Mitochondrial Translation Products
Cells were labeled for 15, 30, 45 or 60 minutes at 37°C in methionine free DMEM medium containing either 150 µCi/ml [35S] methionine (PerkinElmer Life Sciences, Boston, MA) (for cytoplasmic proteins labeling) or 150 µCi/ml [35S] methionine and 100 µg/ml emetine (for mitochondrial proteins labeling) as described (Leary and Sasarman, 2009). Total cellular proteins were extracted in RIPA buffer (1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 150 mM NaCl, 2mM EDTA, and 50 mM Tris-HCl pH 8.0). Proteins were separated by electrophoresis in a 17.5%-SDS-polyacrylamide gel, transferred to nitrocellulose membrane and the labeled mitochondrial translation products were detected by direct autoradiography.

Sucrose Gradient Sedimentation
The sedimentation properties of DDX28 and the ribosomal proteins in sucrose gradients were analyzed essentially as described (Barrientos et al., 2004). Two mg of protein prepared from wild-type HEK293T cells or 400 µg of mitochondria isolated from non-targeting siRNA and siDDX28-treated cells were solubilized in 400 µl of extraction buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 0.5 mM PMSF, EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN), 1% digitonin, and 10 mM MgCl2 or 5 mM EDTA) on ice for 10 min. The clarified extract obtained by centrifugation at 50,000 x g for 15 min was loaded onto a 5 ml linear 0.3 M–1.0 M sucrose gradient containing 20 mM HEPES, 0.5 mM PMSF, protease inhibitor cocktail, 0.1% digitonin, 10 mM $MgCl_2$ or 5 mM EDTA and usually 100 mM KCl. Following centrifugation for 3 h and 10 min at 40,000 r.p.m. using a Beckman 55Ti rotor, the gradients were collected from the bottom in 14 equal fractions and used for immunoblot analysis. For some experiments the mitochondrial extracts were incubated with 600 U/ml RNase A (Fermentas, Thermo Scientific) for 30 minutes at room temperature prior to adding on to the sucrose gradients. To test the salt sensitivity of the DDX28-ribosome interaction, some gradients were performed on extracts prepared in the presence of 150 mM or 300 mM KCl. All of the gradients were performed at least in triplicate using independent mitochondrial preparations. The

gradients reported are representative of each cell line or extraction condition because the patterns observed were reproducible.

RNA isolation and analysis

Total RNA was prepared from whole cells by a modified extraction method with hot acidic phenol (Ausubel et al., 1994) and used for Northern-blot and quantitative RT-PCR analyses. Total RNA was also extracted from sucrose fractions using Quizol and Qiagen RNeasy columns following the instructions of the manufacturer (Qiagen, Boston, MA). For Northern-blot analyses, the RNA extracts were separated on a denaturing 1.8% agarose gel containing 2 M formaldehyde. The quality of the RNA was assessed by staining the gel with ethidium bromide prior to transferring the RNA onto a nylon membrane (Nytran®, SuPerCharge, Schleicher and Schuell, Keene, NH). The RNA bound to the nylon membrane was cross-linked with UV light and pre-hybridized at 65^{o}C for 30 minutes. Subsequently, the specific probes, in a solution containing 7% SDS, 1μM EDTA, 0.5M Na_2HPO_4/NaH_2PO_4, were added to the membrane and was allowed to hybridize overnight at the respective melting temperatures of the probes. The probes were created by PCR on genomic DNA, column-purified (Promega, Madison, WI) and labeled with [α-^{32}P]dATP by random priming. A probe against actin mRNA was used as a loading control. For quantitative RT-PCR analysis of mitochondrial RNAs, total RNA isolated from control and siRNA-treated cells were DNase I treated extensively to remove mtDNA. The absence of mtDNA was verified by PCR. Mitochondrial RNAs were quantified using SYBR Green (Applied Biosystems) and specific primer pairs for each gene following standard procedures. Primers used in this study were: COX1: 5′-CTCTTCGTCTGATCCGTCCT-3′, 5′-ATTCCGAAGCCTGGTAGGAT-3′; 12S rRNA: 5′-TAGAGGAGCCTGTTCTGTAATCGA-3', 5'-TGCGCTTACTTTGTAGCCTTCAT-3', 16S rRNA: 5'-AGAGAGTAAAAAATTTAACACCCAT-3', 5'-TTCTATAGGGTGATAGATTGGTCC-3'; and 18SrRNA was used as a control: 5′-CCAGTAAGTGCGGGTCATAAGC-3′, 5′-CCTCACTAAACCATCCAA TCGG-3′.

Analyzing the DDX28 interactome

A stable pool of HEK293T cells expressing HA-6xHis-tagged DDX28 was established by puromycin (2 μg/ml) selection for 3 weeks after transfection with the construct DDX28-HA-6xHis/pIRESpuro2. Two mg of mitochondria isolated from this cell line or from HEK293T cells were extracted in 500 μl of extraction buffer (20 mM HEPES, pH 7.4, 12.5 mM KCl, 0.5 mM PMSF, EDTA-free protease inhibitor cocktail (Roche), 1% digitonin, and 10 mM $MgCl_2$). Mitochondrial proteins were incubated with α-IgG (control), α-HA or α-DDX28 conjugated Dynabeads (Life Technologies) at room temperature for 4 h. The supernatant containing unbound material was subsequently collected and the beads were washed 3 times with low-salt NET-2 buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% NP-40, EDTA-free protease inhibitor cocktail (Roche)), twice with high-salt NET-2 buffer (with 300 mM NaCl) and once with 1X PBS. In one experiment, the immunoprecipitation was performed using anti-HA antibody-conjugated beads, which were resuspended in 50 μl Laemmli buffer at heated 10 min at 55^{o}C and the collected samples were analyzed by immunoblotting. In another experiment, the immunoprecipitation was performed using anti-DDX28-Prestige antibody-conjugated beads, the bound proteins were eluted using dye-less buffer, precipitated with methanol/chloroform and analyzed by mass spectrometry at the Keck Biotechnology Resource Laboratory (Yale University School of Medicine, New Haven, CT).

RNA-immunoprecipitation

Wild-type mitochondria (2 mg) were submitted to UV-induced RNA-protein cross-linking.

For UV cross-linking mitochondria were exposed for 3X10 min to 254nm UV light in the cold. Mitochondria were subsequently extracted in 100 μl extraction buffer (20 mM Hepes Ph 7.4, 1% digitonin, 25 mM KCl, 1%SDS, 100 U/ml RNase inhibitor, 3 mM vanadylate ribonuclease complex, and yeast protease inhibitor cocktail) on ice for 10 min. The extract was centrifuged at 25,000 g at 4°C for 2X20 min, and the final supernatant was precleared for 1h with noncoated Dynabeads Protein A (Invitrogen) to reduce nonspecific RNA binding to the beads. Binding of anti-DDX28 Prestige antibody (Sigma) or IgG to Dynabeads Protein A was performed according to the manufacturer's instructions. The precleared extracts were then supplemented with 1 ml of extraction buffer without SDS and used in the immunoprecipitation experiment with DDX28-antibody or IgG-crosslinked beads. The immunoprecipitation reaction was performed at room temperature for 1.5 h. To isolate RNA following the immunoprecipitation reaction, the beads were washed five times with extraction buffer without SDS, then incubated for 30 min at 37°C with DNase I, followed by a 30 min incubation at 55°C with Proteinase K in the presence of 0.1% SDS. Samples were then supplemented with EDTA (5 mM) and 1 μg of yeast tRNA. The magnetic beads were then discarded, and RNA was extracted using phenol-chloroform.

Primer extension analysis

Total cellular RNA from control and siDDX28 cells were extracted as described above and was annealed to [^{32}P] 5′-end labeled primer specific to the 5 '-end of the 16S rRNA (p5LSU: 5'- CTGGTAGTAAGGTGGAGTG-3') or to regions near the 16S methylated sites (p1145: 5'-GTTGGTCAAGTTATTGGATCAATTG; p1369/70: 5'-GATCACGTAGGACTTTAATC-3'). The primers were extended with Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT, Promega) according to the manufacturer's protocol. Primer extension products were mixed with loading buffer containing 89% formamide, 4% TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA), 0.12% bromophenol blue, and 0.17% xylene cyanol; separated on 15% polyacrylamide/7 M urea sequencing gel (SequaGel Sequencing System, National Diagnostics); and visualized by either using a PhosphorImager or by exposition to an X-ray film. The same experimental conditions were used with two different dNTPs concentrations (5 and 100 **μ**M) to identify the methylation sites on the 16S rRNA.

Immunocytochemistry

HEK293T cells expressing DDX28-HA were plated on coverslips and grown overnight at 37 °C. Cells were stained for 30 min with 50 nM Mitotracker red (Molecular Probes, Invitrogen), fixed with 2% paraformaldehyde and treated with methanol before incubation with an anti-HA primary antibody (Invitrogen) in 2% BSA. A secondary antibody Alexa Fluor 488 (Molecular Probes, Invitrogen) was used for immunofluorescence detection. Images were obtained in a Leica SP5 confocal microscope (DMI6000 stand), Leica plan apochromat 63×/1.4 numerical aperture objective lens, 60-nm XY pixel size (Nyquist criterion optimized), 2-frame averaging, and sequential scan mode. For Alexa Fluor 488 detection, Argon ion laser, PMT gain 600, 510–540-nm emission band; for Mitotracker Red, 561 nm laser, PMT gain 600, and 580–630-nm emission band.

Miscellaneous Procedures

Standard procedures were used for the preparation and ligation of DNA fragments, and for transformation and recovery of plasmid DNA from *E. coli* (Sambrook et al., 1989). Yeast were transformed as described (Schiestl and Gietz, 1989). Protein concentration

was measured with the Folin phenol reagent (Lowry et al., 1951). Proteins were separated by SDS-PAGE in the buffer system of Laemmli (Laemmli, 1970), and membranes with immobilized proteins were treated with antibodies against the appropriate proteins followed by a second reaction with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO). The Super Signal chemiluminescent substrate kit (Pierce, Rockford, IL) was used for the final detection.

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