Supplementaly Data-

”p32/gC1qR is indispensable for fetal development and mitochondrial translation: importance of its RNA binding ability” Yagi et al.

Supplementary Method and Figure legends, Supplementary Figure S1-S7, Supplementary Table S1

Supplemental Methods

Generation of p32-deficient cell lines and mice

To generate the p32 targeting vector, DNA fragments flanking exons 1–2, with a XhoI–KpnI fragment (3.2 kb) as the 5′ arm and a SalI–SacII fragment (4.4 kb) as the 3′ arm, were isolated from a 129/Sv genomic library and subcloned into a pflox vector. Flanked loxP sites and a neomycin-resistance cassette were introduced as shown in Sup plemental Figure S1A. Embryonic stem (ES) cells were transfected with a linearized targeting vector by electroporation, and then selected in medium containing 200 μg/ml G418 (Sigma). Of the 96 G418-resistant ES cell clones analyzed, 12 showed correct targeting. One $p32^{flox/+}$ ES cell clone was injected into C57BL/6J blastocysts to obtain chimeric mice. Chimeric males that transmitted the targeted allele to the germline were mated with C57BL/6 females, and germline transmission of the targeted allele was confirmed by Southern blotting and PCR analysis. $p32^{flox/+}$ mice were crossed with a heterozygous C57BL/6 EIIa-cre deleter strain that expresses Cre recombinase after fertilization (in the embryo before implantation) to generate $p32^{+/-}$ heterozygotes. Heterozygous siblings were then intercrossed.

$p32^{flox/flox}$ mouse embryonic fibroblasts (MEFs) were prepared from E13.5 $p32^{flox/flox}$ embryos. $p32^{-/-}$ MEFs were generated by the expression of Cre recombinase in $p32^{flox/flox}$ MEFs. $p32^{-/-}$ MEFs were then immortalized by expression of the SV40 large T antigen. Pregnant mice were intraperitoneally injected with bromodeoxyuridine (BrdU; 100 μg/mg of mouse body weight) at 2 h before sacrifice. Whole embryos were photographed in phosphate-buffered saline (PBS) using a microscope-mounted digital camera (Leica). Embryonic stages were estimated by timed pregnancies and somite counts. Embryos were fixed in 4% paraformaldehyde, pH 7.4, dehydrated in ethanol and then paraffin-embedded. Specimens were sectioned at 5 μm and stained with hematoxylin-eosin or processed for immunohistochemistry.

PCR genotyping and Southern blotting

Mouse tail genomic DNA was isolated by phenol-chloroform extraction after

cell lysis with Triton X-100 and digestion with proteinase K. Primer sequences are listed in Supplemental Table S1. The length of p32 PCR products from wild-type and knockout cells was ~2500 and ~340 bp, respectively.

For Southern blotting, mouse embryo genomic DNA was isolated by digesting cells in lysis buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml proteinase K) for 16 h at 37°C, and then spooling genomic DNA after adding an equal volume of isopropyl alcohol. DNA was digested overnight with BamHI and XhoI, size-fractionated by 0.9% agarose gel electrophoresis and then transferred to a Hybond-N membrane (GE Healthcare) overnight, according to the manufacturer's instructions. After rinsing with 6× saline-sodium citrate, membranes were UV cross-linked (Stratalinker®; Stratagene). A probe designed from the region downstream of exon 6 (1200 bp) was nick translated in the presence of ^{32}P-α-cytidine triphosphate, and unincorporated nucleotides were removed by gel filtration (Biospin G50; GE Healthcare). Hybond membranes were exposed to X-ray film with intensifying screens for 2–5 days.

Immunohistochemical analysis of mouse embryo sections

Mouse embryo tissue was fixed with 10% buffered formalin and embedded in paraffin. Sagittal sections (5 μm thick) were cut and mounted on APS-coated glass slides. After routine deparaffination and re-hydration via an ethanol gradient, slides were steam heated for 20 min to expose the antigen. Endogenous peroxidase activity was quenched using 3% (v/v) H_2O_2, followed by three 5 min PBS washes, and then sections were blocked with Blocking One (Nakalai Tesque). Specimens were incubated for 1 h with the anti-p32 antibody diluted at 1:200 in PBS containing 0.1% (w/v) BSA, followed by three 5 min PBS washes, and then incubation with Histofine Simple Stain Mouse MAX-PO-rabbit (Nichirei) for 15 min. Specimens were stained with Simple Stain DAB Solution (Nichirei) for 3 min and counterstained with hematoxylin for 30 s. Sections were immediately dehydrated by sequential immersion in a gradient of ethanol and then xylene, followed by mounting with Permount (ProSciTech) and then coverslipped. Images were obtained using an HS all-in-one fluorescence microscope (BZ-9000; Keyence).

HeLa cell culture

HeLa human cervical carcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 2% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. To generate stable inducible cell lines, HeLa

Flp-In™ T-Rex™ cells (Invitrogen) were co-transfected with pcDNA5/FRT/TO harboring p32-HA and pOG44 using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. After transfection, cells were cultured in DMEM containing hygromycin B (100 μg/ml) and blasticidin S (10 μg/ml) (Invitrogen). After selection for approximately 3–4 weeks, clonal foci were identified, transferred into separate wells and checked for expression using the anti-HA antibody (Invitrogen). Expression was induced by the addition of doxycycline (1 μg/ml) to culture medium for the indicated time.

Recombinant proteins

Recombinant proteins were expressed in *Escherichia coli* BL21(DE3). His-p32 and His-p32 (K89A/K93A) proteins were purified as a His-tag fusion protein. Briefly, the His-tag fusion protein expressed in bacteria was induced by incubation with 1 mM isopropyl β-D-1-thiogalactopyranoside, and then the cells were lysed by sonication in 1 ml binding buffer (1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1× proteinase inhibitor cocktail (EDTA-free) (Roche) and 1% Triton-X 100 in PBS, pH 7.4). Cell debris was removed by centrifugation, and the supernatant was subjected to affinity column chromatography using a Nickel Sepharose 6 Fast Flow (GE Healthcare) according to the manufacturer's instructions.

IP after crosslinking for liquid chromatography and mass spectrometry (LC/MS/MS)

All procedures were performed at 4°C or on ice. Two milligrams of mitochondrial protein were resuspended in 1 ml crosslinking buffer (0.25 M sucrose, 20 mM HEPES-KOH, pH 7.4, 2 mM EDTA and 25 mM NaCl). Then, 1% formaldehyde was added, and the mixture was kept on ice for 30 min. After incubation, 150 mM glycine was added to quench the crosslinking reaction, and the sample was centrifuged at 10,000 × *g* for 6 min to precipitate the mitochondria. The mitochondria were solubilized with 100 μl IP buffer (as described above except containing 1% SDS). After a 10-fold dilution with 1 ml IP buffer, the IP reaction was performed as described above. Immunoprecipitates were heated at 95°C for 30 min to cleave the crosslinks, and proteins were separated by 12% SDS-PAGE. Bands were visualized by staining with Coomassie brilliant blue (CBB). Immunoprecipitates were separated by SDS-PAGE and immunoblotted with each antibody.

In-gel protein digestion and LC/MS/MS

A CBB-stained band was excised, and the protein was digested with trypsin (Wako) for 12 h in digestion buffer (100 mM Tris-HCl, pH 9.0, 0.5% SDS and 1 mM EDTA). The supernatant containing the digested peptides was analyzed by LC/MS/MS (1100 Series; Agilent). Peptide masses were compared with those in the peptide mass databases using the MASCOT program (Matrix Science). All proteins in the SWISS-PROT mammalian database were included. The peptide mass error was limited to 1.0 Da and the MS/MS mass error was limited to 0.8 Da. Only one missed cleavage was accepted.

Supplemental Figure legends

Supplemental Figure S1 Genotypic analysis of p32$^{-/-}$ embryos.

(A) Strategy of p32 gene targeting. The top bar indicates the enzymatic cleavage sites at the p32 genomic locus. Homologous recombination using this construct (middle bar) resulted in a knockout allele (bottom bar) in which a large part of exon 3 was replaced by the loxP-flanked PGK promoter-driven neomycin resistance cassette (white segment - Neo; loxP sites). The approximate location of *BamHI* and *XhoI* restriction endonuclease sites used for Southern blot analysis are also shown. LoxP sites are indicated by triangles flanking exon 3 and pgk-neo genes. The position of the external probe for Southern blotting is indicated as a bar (probe). The arrows are PCR primers used for genotyping.

(B and C) Homologous recombination in ES cell clones was confirmed by Southern blotting and PCR analysis. (B) Genomic DNA isolated from ES cells and tail biopsies was digested with *BamHI* and *XhoI*, hybridized with an external probe and analyzed by autoradiography. (C) Genotype analysis of ES cells by PCR using fo-2 and re-2 primers. The band is specific for targeting alleles (fl/+).

(D) PCR-based genotyping strategy. PCR analysis of genomic DNA from *p32*$^{+/+}$, *p32*$^{+/-}$ and *p32*$^{-/-}$ embryos was performed using primers fo-1 and re-1 between loxP sites. Data are representative of at least three mice analyzed for each phenotype.

Supplemental Figure S2 Establishment of p32-knockout MEFs.

Upper panel: western blot indicating p32 expression in wild-type (+/+) and p32-deficient (–/–) MEFs (two independent cell lines for each) compared with that of β-actin as a loading control. Lower panel: microscopic analysis of MEFs. Scale bar: 50 μm.

Supplemental Figure S3 Re-expression of p32, and molecular analysis of mitochondria in p32$^{-/-}$ MEFs.

(A) Schematic representation of the primary structure of p32. The number of amino acid residues on the boundaries is indicated. The location of the mitochondrial targeting signal is indicated as MTS.

(B) Cell lysates from p32$^{+/+}$ (wild-type) and p32$^{-/-}$ MEFs that were transfected with various constructs were analyzed by immunoblotting with anti-p32 and anti-β-actin antibodies.

(C) Treatment of mature p32-transfected cells with the proteasome inhibitor MG 132

(10 μM, 24 h) significantly increased the expression levels of mature p32 (two independent clones).

Supplemental Figure S4 EDTA treatment decreased 55S mitoribosomes

Upper panels: Sedimentation analysis of mitoribosomal particles by centrifugation via a linear 15–30% sucrose density gradient. Fraction numbers are indicated. The migration of mitoribosomal particles in wild-type (+/+) MEFs and EDTA treated MEF cells was determined by immunoblotting with antibodies against MRPL3 (39S large subunit), MRPS29 and S22 (28S small subunit), and p32. Arrows indicate peaks of optical density for the S value markers.
Lower panels: Representative blots were analyzed densitometrically. The signal intensity of the protein in each fraction was plotted. The maximum value was 100% for each protein level.

Supplemental Figure S5 p32 interaction with RNA

Co-IP of RNA visualized on an agarose gel in HeLa cells. RNA from whole cell lysates (lanes 1 and 2) and immunoprecipitants with anti-HA (lanes 3 and 4) were reverse transcribed and then amplified by PCR. The PCR products were loaded on 2% agarose gels.

Supplemental Figure S6 Size exclusion chromatography analysis

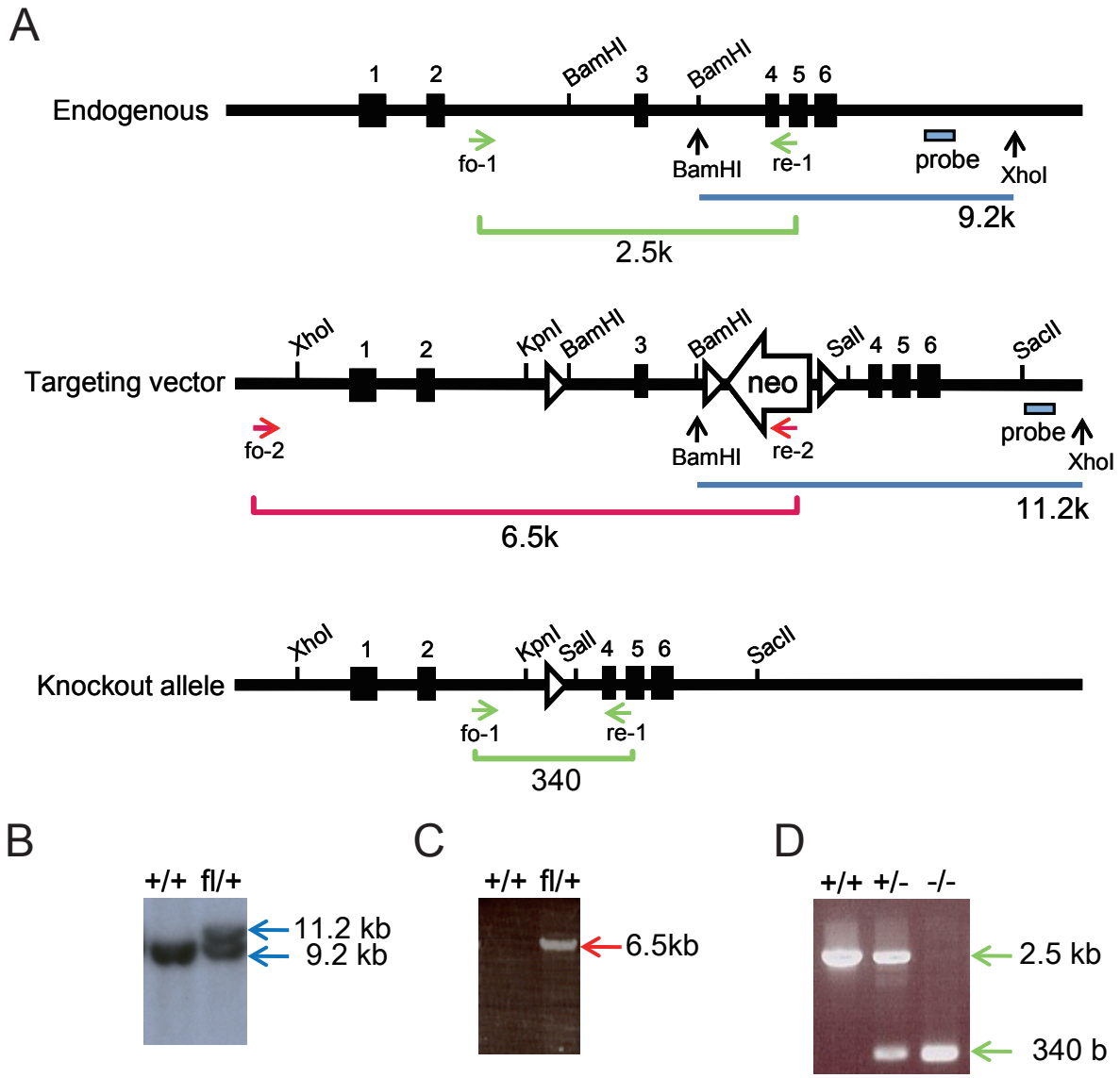
The purified wild and mutant (K89A/K93A) p32 were analyzed by size exclusion chromatography analysis. Both recombinant proteins were detected at almost 90kDa.

Supplemental Figure S7 p32 interacts with mitochondrial maintenance factors *in vivo*.

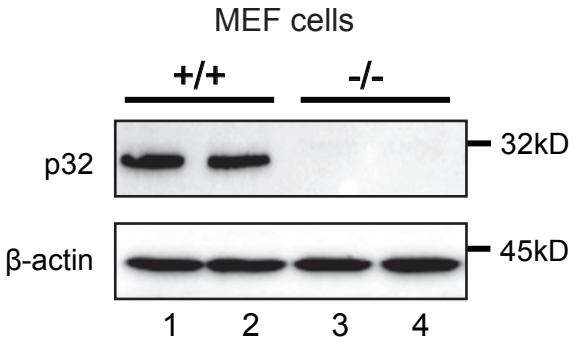
Mitochondria from HeLa cells with DOX(–) and DOX(+) were subjected to crosslinking. Lysed mitochondria were immunoprecipitated with anti-HA agarose, resolved by SDS-PAGE and stained by CBB. p32-HA is indicated by major arrows. Proteins were identified by LC/MS/MS and showed the Table 2.

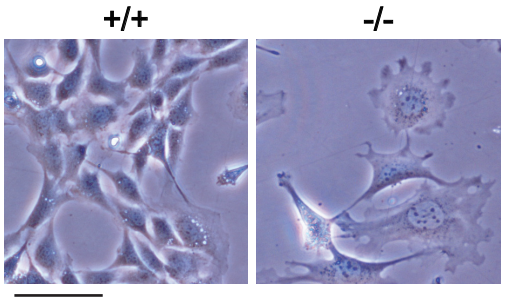
Supplemental Table S1 List of primers in this experiment.

Supplementary Figure S1

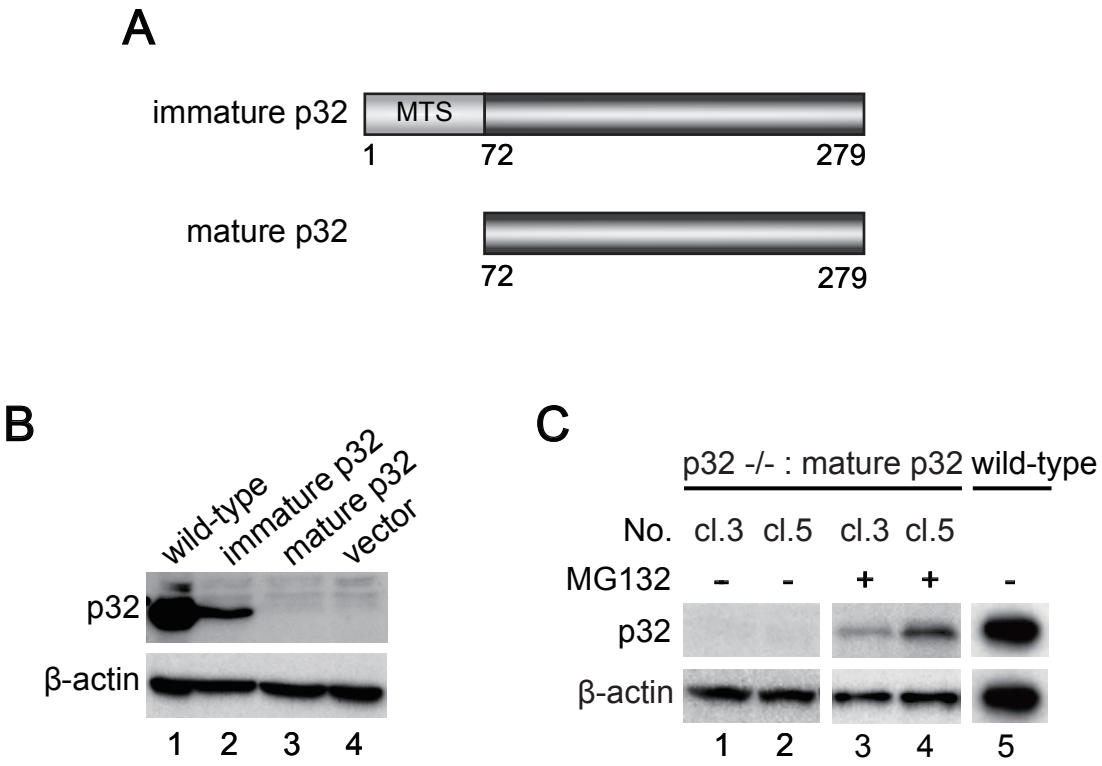


Supplementary Figure S2

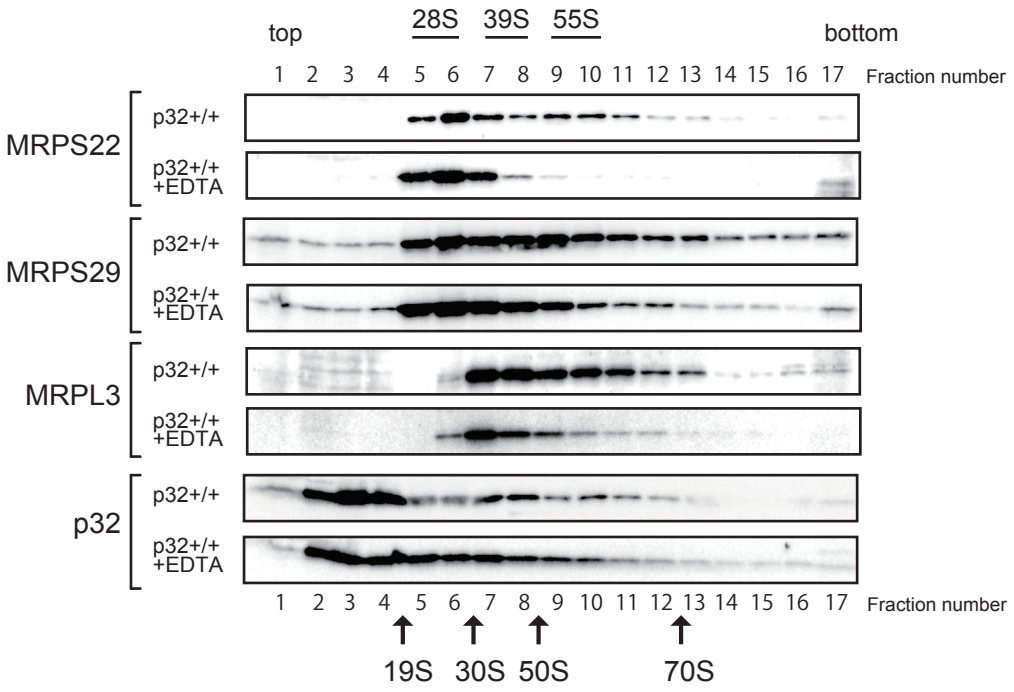


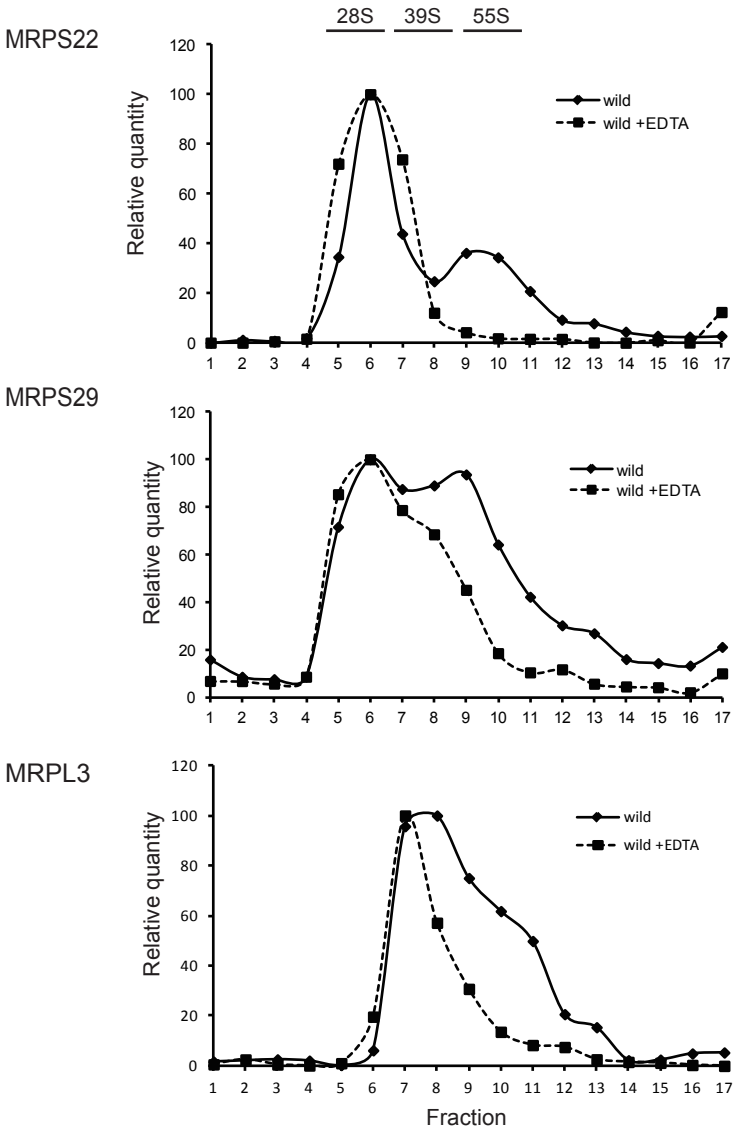


Supplementary Figure S3

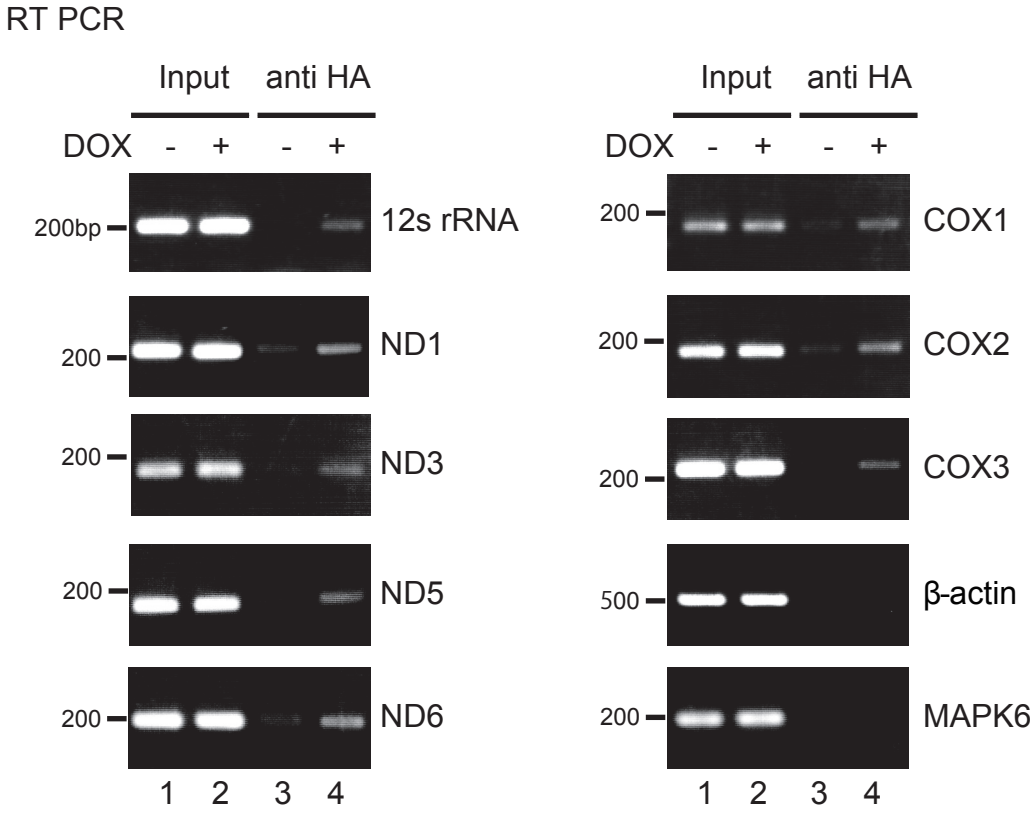


Supplementary Figure S4

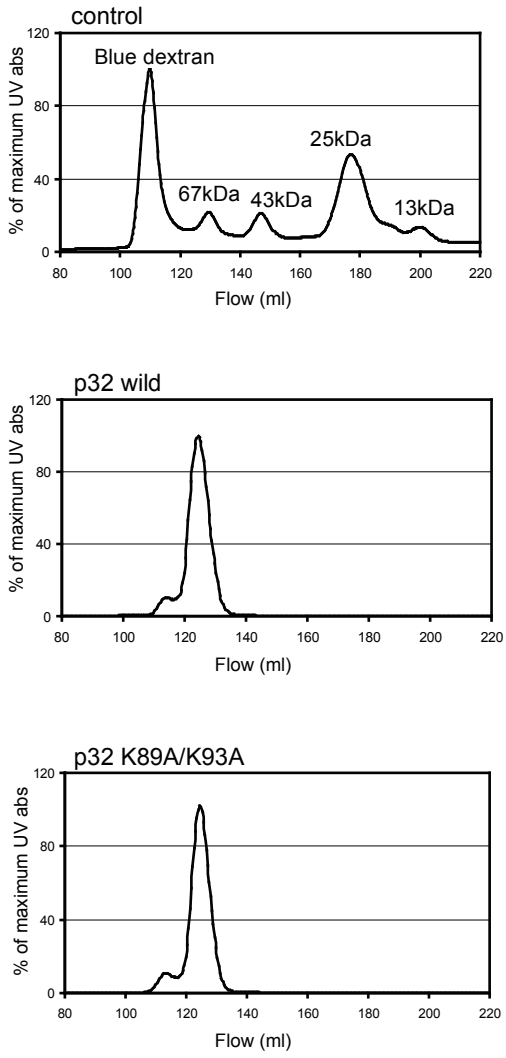




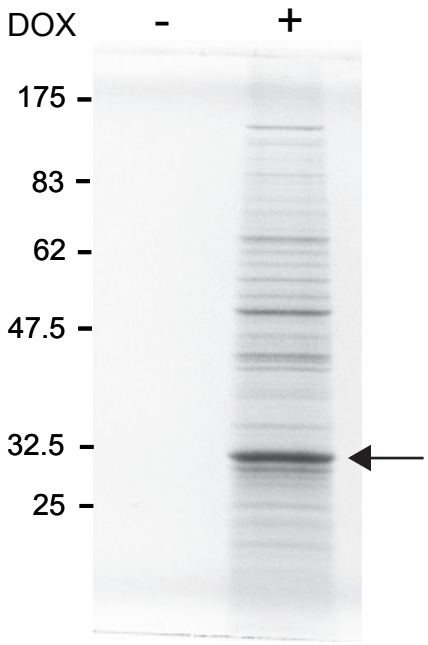
Supplementary Figure S5



Supplementary Figure S6



Supplementary Figure S7



Supplemental Table S1.Primer sequences

Knockout allele genotyping and Southern blotting primers

Primer name	Sequence 5'→3'
Genotyping fo-1	CTCCCATCTGGCTTGTGTTT
Genotyping re-1	TTCCCCTCTGACTGTTTTGG
Genotyping fo-2	GAACTCCCATCTGGCTTGTGTTTG
Genotyping re-2	TTGGGAAGACAATAGCAGGCATGC
Southern probe-fo	CTAAATCCTGATGCCAACCC
Southern probe-re	AGGAGAACGTTCTATTATGGG

Knockout mouse cloning primers

Primer name	Sequence 5'→3'
Cloning F1-fo	GCAATTCTCGAGTGCTGCAGGGGTTTATCTT
Cloning F1-re	GCAATTGGTACCCCAAGAGTCAGAGGCTGGT
Cloning F2-fo	CCAGCCTCTGACTCTTGGT
Cloning F2-re	TTCCCCTCTGACTGTTTTG
Cloning F3-fo	AAATTTCCGCGGCTGGGCTTATAGGCTTGCAC
Cloning F3-re	AAATTTGTCGACCAAAACAGTCAGAGGGGAA

qPCR primers (mouse)

Primer name	Sequence 5'→3'
12S rRNA-fo	CCGCTCTACCTCACCATCTC
12S rRNA-re	CCCATTTCATTGGCTACACC
16S rRNA-fo	GGGATAACAGCGCAATCCTA
16S rRNA-re	GATTGCTCCGGTCTGAACTC
ND1-fo	GGATCCGAGCATCTTATCCA
ND1-re	GGTGGTACTCCCGCTGTAAA
ND2-fo	AGGGATCCCACTGCACATAG
ND2-re	CTCCTCATGCCCCTATGAAA
ND3-fo	TTCGACCCTACAAGCTCTGC
ND3-re	TGAATTGCTCATGGTAGTGGA
ND4-fo	CCACTGCTAATTGCCCTCAT
ND4-re	CTTCAACATGGGCTTTTGGT
ND5-fo	TCCTACTGGTCCGATTCCAC
ND5-re	TTTGATGTCGTTTTGGGTGA

ND6-fo	CGATCCACCAAACCCTAAAA
ND6-re	TTGGTTGTCTTGGGTTAGCA
COXI-fo	GGTCAACCAGGTGCACTTTT
COXI-re	TGGGGCTCCGATTATTAGTG
COXII-fo	ACGAAATCAACAACCCCGTA
COXII-re	GGCAGAACGACTCGGTTATC
COXIII-fo	CAAGGCCACCACACTCCTAT
COXIII-re	ATTCCTGTTGGAGGTCAGCA
ATP6-fo	CCTTCCACAAGGAACTCCAA
ATP6-re	GGTAGCTGTTGGTGGGCTAA
Cytb-fo	TGAGGGGGCTTCTCAGTAGA
Cytb-re	CTGTTTCGTGGAGGAAGAGG
β-actin-fo	TGTTACCAACTGGGACGACA
β-actin-re	GGGGTGTTGAAGGTCTCAAA
18S rRNA-fo	CGCGGTTCTATTTTGTTGGT
18S rRNA-re	AGTCGGCATCGTTTATGGTC
HPRT-fo	AAGCTTGCTGGTGAAAAGGA
HPRT-re	TTGCGCTCATCTTAGGCTTT

qPCR primers (human)

Primer name	Sequence 5’→3’
12S rRNA-fo	AAACTGCTCGCCAGAACACT
12S rRNA-re	CATGGGCTACACCTTGACCT
16S rRNA-fo	GCTAAACCTAGCCCCAAACC
16S rRNA-re	TTGGCTCTCCTTGCAAAGTT
ND1-fo	ATGGCCAACCTCCTACTCCT
ND1-re	GCGGTGATGTAGAGGGTGAT
ND2-fo	AAGCAACCGCATCCATAATC
ND2-re	TCAGAAGTGAAAGGGGGCTA
ND3-fo	ACCACAACTCAACGGCTACA
ND3-re	TTGTAGGGCTCATGGTAGGG
ND4-fo	CCTGACTCCTACCCCTCACA
ND4-re	ATCGGGTGATGATAGCCAAG
ND5-fo	ACATCTGTACCCACGCCTTC
ND5-re	TCGATGATGTGGTCTTTGGA

ND6-fo	TGATTGTTAGCGGTGTGGTC
ND6-re	CCACAGCACCAATCCTACCT
COXI-fo	GGCCTGACTGGCATTGTATT
COXI-re	TGGCGTAGGTTTGGTCTAGG
COXII-fo	TTCATGATCACGCCCTCATA
COXII-re	TAAAGGATGCGTAGGGATGG
COXIII-fo	CCCGCTAAATCCCCTAGAAG
COXIII-re	GGAAGCCTGTGGCTACAAAA
ATP6-fo	TATTGATCCCCACCTCCAAA
ATP6-re	GATGGCCATGGCTAGGTTTA
Cytb-fo	TATCCGCCATCCCATACATT
Cytb-re	GGTGATTCCTAGGGGGTTGT
MAPK6 -fo	GAAGAAGCACTCTCCCATCCT
MAPK6-re	ATGTACAGGCCAATCATGCTC

RNA binding assay primers

Name	Sequence 5’-3’
14 mer RNA	GUUUACGGUGCUA
22 mer poly A	AAAAAAAAAAAAAAAAAAAAAA
22 mer poly U	UUUUUUUUUUUUUUUUUUUUUU
40 mer RNA	GGGAGAAAAACAAAACAAAACAAAACUAGCACCGUAAAGC