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Supplementary Methods

Generation of mice carrying a *Clp1*^{K127A} allele. All DNA fragments for the construction of the *Clp1* targeting vector were derived from BAC RP23-387F9 either by restriction digest or PCR. For the short arm, a 842 bp NdeI/SacI fragment was sub-cloned into pBSII, and cloned into the HindIII/EcoRV sites of the plasmid pLFNeo-DTA upstream of the DTA cassette. For construction of the middle arm, a 1512 bp SpeI/NdeI fragment encompassing exon 2 of *Clp1* was sub-cloned into pBSII. Site-directed mutagenesis of lysine-127 to alanine was generated by PCR with primers 5'-CCT ACT GAT GTG GGC GCG TCC ACT GTG TGT C-3' and 5'-CGA CAC ACA GTG GAC GCG CCC ACA TCA GTA G -3' and the mutated PCR fragment was cloned into the NheI site of pLFNeo-DTA just upstream of the *neo* selection cassette. For the long arm, a 9.7 kb KpnI/SpeI fragment encompassing exon 1 was sub-cloned into pBSII, and cloned into the SacII sites of pLFNeo-DTA upstream of the *neo* selection cassette. The targeting vector was linearized with XhoI and electroporated into A9 embryonic stem (ES) cells. A9 ES cells were derived in-house from the 129/BL6 mixed strain. Approximately 2-3 in 100 ES cell clones were identified as correctly targeted by genomic Southern blot (BamHI digest of genomic DNA, probed with a 828 bp fragment 5' to the short arm). Two A9-derived ES cell clones were injected into C57BL/6 blastocysts. Chimeric mice derived from both independent A9-derived ES cell clones transmitted the mutant allele through the germ line. Mice carrying the *frt*-flanked PGK-*neo* cassette were crossed to FLPe transgenic mice to excise the PGK-*neo* cassette and subsequently crossed to C57BL/6 mice to remove the FLPe recombinase transgene to generate *Clp1*$^{K/+}$ mice. Genotypes were confirmed by Southern blot using the probe indicated above. Mice carrying the mutant *Clp1* allele were backcrossed five times to C57BL/6, Balb/c, or CBA/J mice. Of note, on a CBA/J background, *Clp1*$^{K/K}$ pups born to older mothers were more prone to die at birth than pups born to younger moms. Both *Clp1*$^{K/+}$ male and *Clp1*$^{K/+}$ female mice were viable and exhibited normal fertility. For mouse genotyping, genomic DNA from mice tails was isolated and amplified by PCR with two primers:

P1, 5'-TTG GTT CAG GTA TTA AGT CGT TGG-3';

P2, 5'-GAA TTG CAT AGT CTT TCC TCC ATC-3';

PCR products were 207 bp (wild type allele) and 360 bp (mutant allele). P53-deficient mice (C57BL/6J-Trp53 tm1Tyj)[1] and Hb9:GFP transgenic mice (B6.Cg-Tg(Hlxb9-GFP)1Tmj/J)[2] were obtained from The Jackson Laboratory. All mice were maintained in a pathogen-free environment according to institutional guidelines.

Generation of Hb9 transgenic mice. pHb9-MCS-IRES-eGFP was purchased from Addgene (Addgene plasmid 16283). It contains a 9 kb fragment of the Hb9 promoter, a 5′ splicing substrate, an internal ribosome entry sequence (IRES), enhanced green fluorescence protein (EGFP), and a bovine polyadenylation (polyA) signal[3]. The cDNA encoding the Flag-tagged mouse CLP1 was cloned into the PmeI site, resulting in the expression construct pHb9:Flag-CLP1-IRES-eGFP. To produce transgenic founders, the plasmid was first linearized and vector sequences removed using XhoI, and then purified using GENECLEAN SPIN Kit (Q-Biogene). Linearized DNA was injected into the pronucleus at IMBA Transgenic Service Core Facility. Founders were screened by PCR to identify carriers of exogenous Flag-CLP1 cDNA. Genotyping was performed by PCR with two primers resulting in a 406 bp PCR product:

5′ -ACA CTC ATA CAG CCT TGG AGC AG-3′ and

5′ -GTG TTG ATG ACA CAG CCA CTC AC-3′ .

Of note, rescue of the phenotype was also observed using a second Hb9-CLP1 transgenic mouse line (Supplementary Fig. 31b, and not shown).

Western blot. Western blot was carried out with antibodies reactive to phospho-p53 (serine 18; Cell signaling), p53-CM5 (Leica), p21 (BD Biosciences), c-myc (Sigma), Flag M2 (Sigma), β-actin (Sigma), GFP (Invitrogen) and surfactant protein A (Abcam). In addition, rabbit polyclonal antibodies (Gramsch Laboratories, Schwabhausen, Germany) were raised against peptides of human TSEN2 (N-NGDSGKSGGVGDPREPLG-C), TSEN34 (N-AKKQKLEQASGASSSQEAGS-C), TSEN54 (N-RSRSQKLPQRSHGPKDFLPD-C), and mouse CLP1 (N-VVERSKDFRREC-C). Sera were subsequently affinity purified for Western blot analysis. Samples for Western blot were lysed in lysis buffer (0.5% NP-40, 10% glycerin, 50 mM HEPES-KOH (pH 7.8), 150 mM NaCl, 1 mM EDTA) with protease and phosphatase inhibitor cocktail (Thermo scientific). Total protein was separated by SDS–PAGE, transferred to Immobilon-P membranes (Millipore) and probed with the antibodies described above using

standard protocols. For semi-quantitative Western blotting[4,5], we used serial dilutions of cell lysates to create a standard curve for phosphorylated p53 (S18) and β-actin protein level quantification. The images, in which the intensity of bands was within the linear range, were used for quantification. Quantification of pixel intensity was performed with the MetaMorph software (Molecular Devices) after performing background subtraction and data normalized to β-actin loading controls. The intensity of normalized phosphorylated p53 (S18) at 1 hr for $Clp1^{+/+}$ cells was arbitrarily set at 1.0.

Immunohistochemistry. Diaphragm muscles were stained as described previously[6]. Briefly, dissected and fixed muscles were stained overnight at 4°C with antibodies against neurofilament (Millipore) and S100 (DAKO) or synaptophysin (Zymed) in 2% BSA, PBS, 0.5% Triton X-100, washed three times for 20 minutes in 0.5% Triton X-100/PBS and incubated overnight at 4 °C with Alexa 488-conjugated goat anti-rabbit IgG and Alexa-594-conjugated α-Bungarotoxin (α-BGT; Molecular Probes). Muscles were washed twice for 20 minutes in 0.5% Triton X-100/PBS, twice in PBS and postfixed in 1% paraformaldehyde (PFA), rinsed in PBS and flat mounted in Vectashield (Vector Labs). The stained muscles were visualized by using a Leica TCS SP5 spectral confocal microscope with HCX PL APO CS 10x / 0.4 or HCX PL APO CS 63x / 1.4 oil objectives. Lumbar spinal cords from embryonic, neonate and adult mice were carefully dissected, fixed in 4% PFA overnight, washed with PBS and kept for 24 hrs in 0.1 M phosphate buffer containing 30% sucrose. The tissues were finally embedded in OCT compound and rapidly frozen on dry ice. Serial sections of 10-μm thickness were cut with a cryostat. ChAT immunoreactivity was detected with goat anti-ChAT (Chemicon International) diluted 1/50 and followed by Alexa 488-coupled donkey anti-goat IgG or Alexa 594 coupled donkey anti-goat IgG diluted 1/500 (Molecular probe). SMI-32 antibody (Covance) against non-phosphorylated neurofilament was used for spinal motor neuron staining. The antibody diluted 1/500 and followed by Alexa 594-coupled donkey anti-mouse IgG diluted 1/500. The eGFP signal was enhanced by staining with an anti-GFP antibody (Invitrogen) and Alexa 488 coupled donkey anti-rabbit IgG. All motor neuron counts were performed in a blinded fashion. We counted the numbers of motor neurons in every fifth section of L5 or C4-C5. Sections were viewed and photographed with an LSM 510 laser scanning confocal microscope (Carl Zeiss). Lung tissues were fixed overnight (16–18 hrs) in 4% paraformaldehyde in PBS and then

dehydrated and embedded in paraffin. We used 5-μm paraffin sections for standard hematoxylin and eosin (H&E) staining or immunohistochemical staining. We performed antigen retrieval (Vector Lab H-3300) and then used an R.T.U. Vectastain Universal Elite ABC Kit and NovaRED or via-in-pad (VIP) substrate (all from Vector Lab) following the manufacturer's protocols. The primary antibodies used were Caveolin-1 (Cell signaling) and Surfactant protein-C (Santa Cruz FL-197). Sections were viewed and photographed with an Axioskope 2 plus light microscope (Carl Zeiss) and Image access (Imagic Bildverarbeitung).

Quantification of NMJ morphology. Endplate area was quantified by delineating the outside edge of α-bungarotoxin labeled NMJs, then measuring the total area within that outline using ImageJ. AChR surface area was quantified from 3D reconstructions using Imaris (Bitplane). Of note, *Clp1*$^{K/K}$ mice exhibited normal formation of muscle groups in the front and hind limbs.

Footprint test. The hind limb paws were treated with blue ink (Pelikan) and mice were allowed to walk over a white paper strip in a straight-alley runway towards an enclosed goal box. Tracks were collected, and the hind limbs stride length was calculated and analyzed. Of note, the head to anus length of the mice was comparable, i.e. the impaired stride length is not due to a smaller body size.

Hanging wire test. The mice were placed on the top of a cage, which was then inverted and suspended above the home cage; the latency until the animal falls was recorded. This test was performed three days per week with three trials per session. The average performance for each session is presented as the average of the three trials.

Rotarod test. The Rotarod test was performed using an Ugo Basile 7650 accelerating RotaRod (Ugo Basile Srl). The mouse was placed onto a rotating rod. Fixed-speed rotarod testing was carried out at a speed of 4 rpm to a maximum time of 300 seconds. For the accelerating task, the Rotarod was accelerated from 4 to 40 rpm/minutes. The latency to fall was recorded for each of the three trials and averaged to generate the overall time for each mouse. Animals rested a minimum of 30 min between trials to avoid fatigue.

Cell Culture. Primary MEFs were obtained from day 13.5 embryos and immortalized by the 3T3 protocol. HeLa cells, primary and immortalized 3T3 MEFs were grown in complete medium (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO-BRL). NSC-34 mouse motor neuron cell line was kindly provided by Neil Cashman[7]. Treatment of cells was performed with following reagents, all purchased at Sigma-Aldrich, at concentrations indicated in the respective figure legends: staurosporine, actinomycin D, cycloheximide, α-amanitin, glucose oxidase, menadione, paraquat dichloride, nefazodone hydrochloride, camptothecin, FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), rotenone, puromycin and human recombinant Angiogenin. The viability of *Clp1*$^{K/K}$ MEFs was comparable to that of control MEFs in response to serum starvation, glucose withdrawal, and UV irradiation (not shown), indicating that *these cells* do not exhibit a global hypersensitivity to diverse cell death triggers.

Sensory behavioral analyses. For the hot plate assay, age and sex-matched *Clp1*$^{K/K}$ and wild type littermates mice were acclimated to the hot plate apparatus (Ugo Basile) and then tested for hot plate latency at 55ºC. Jumping, biting, licking, and clutching of hind paws were considered a nociceptive response as described previously[8]. For the tail-flick test, a light beam was focused onto the tip of the tail, and the latency to tail withdrawal was taken as a measure of the nociceptive threshold to radiant heat (Harvard Apparatus). The mechanical pain test was performed by applying von Frey hairs to the dorsal surface of each hindpaw until a hindlimb withdrawal response was observed; the hair with the minimum bending force required to produce a nociceptive response was recorded. Intraplantar injection of capsaicin that preferentially stimulates C fibers[9] was used to assess chemical pain. Capsaicin (1 µg per 15 µL) was injected into the plantar surface of the left hind paw. The duration of nociceptive responses (licking, biting and shaking) was recorded for 3 min.

Metabolic studies. All measurements were performed at room temperature (21°C-23°C) in a PhenoMaster System (TSE systems, Bad Homburg, Germany) using an open circuit calorimetry system. Mice were housed individually, trained on drinking nipples for 72 hrs and allowed to adapt to the PhenoMaster chamber for at least 24 hrs. Food and water were provided *ad libitum*

in the appropriate devices and measured by the build-in automated instruments. Parameters of indirect calorimetry, activity, food and water intake were measured for 5 consecutive days. The cold exposure experiment was performed as follows: mice were kept at 4°C for 1 hr and then kept at room temperature for 30 min. Rectal body temperatures were measured at 0, 60, 75 and 90 min using a Microprobe thermometer (World Precision Instruments).

Sciatic nerve analyses. Sciatic nerves were dissected and fixed in glutaraldehyde and chromeosmium, dehydrated in ethanol and embedded in Epon 812 (Serva Electrophoresis GmbH). Then, 1-µm-thick sections were cut with an ultra-microtome and stained with toluidine blue. The cross-sectional area of the nerves and the diameter and density of individual nerve fibres within the nerves was determined using an Axioskop microscope and Axiovision software (Carl Zeiss). For immunohistochemical analysis of the sciatic nerve of adult mice, animals were perfused with 4% buffered formalin. Sciatic nerves were embedded in paraffin and 3 µm thick sections were stained following standard immunohistochemistry procedures using the Ventana Benchmark XT machine (Ventana, Tucson, Arizona) with antibodies against amyloid precursor protein (APP; 1:3000, Millipore, Hofheim, Germany) followed by detection with Mouse Stain Kit (Nichirei Biosciences, Tokyo, Japan). Visualization of secondary antibodies was performed using the "Ultra View Universal DAB Detection Kit" from Ventana.

Histologic and morphometric analysis of the gastrocnemius muscle. For histologic analysis, 8 µm-thick sections were prepared from gastrocnemic muscles that had previously been frozen in liquid nitrogen-cooled 2-methylbutane. H&E staining was performed following routine protocols. The mean muscle fibre diameter was determined using an Axioskop microscope and Axiovision software (CarlZeiss Microimaging, Göttingen, Germany). In each experimental group, n=3 mice were analysed and at least 100 randomly selected muscle fibres were measured in each mouse.

Dorsal root ganglion (DRG) explant cultures. Dorsal root ganglion explants of E13.5 mice were cultured on poly-D-lysine (PDL)-laminin-coated glass coverslips. The explants were grown in Neurobasal medium supplemented with 2% B-27, 1% glutamine, 1% penicillin–streptomycin, and 12.5 ng/mL mNGF 2.5S (Alomone Labs; N-100) for 24 hours. The culture

was then fixed with 4% paraformaldehyde and 15% sucrose and immunostained for neuronal class III β-tubulin (Tuj1; Covance; MRB-435P; 1:500–1:2000).

Lineage conversion of MEFs into induced motor neurons. MEFs were isolated from *Clp1*$^{+/+}$ and *Clp1*$^{K/K}$ MEFs. MEFs were converted into induced motor neurons as described[10]. Briefly, retroviruses encoding *Isl1*, *Lhx3*, *Ngn2*, *Hb9*, *Ascl1*, *Brn2*, and *Myt1l* were produced by transfecting each cDNA in a pMXs backbone into Plat-E cells. The supernatants were collected at 48 and 72 hrs and added to 75,000 MEFs per 35mm well in an equimolar ratio in the presence of 5 μg/ml polybrene. Starting two days after transduction, N3 medium (DMEM/F12, N2, B27, glutamax, penicillin/streptomycin (all Invitrogen)) + 10 ng/ml GDNF, CNTF, and BDNF was used to culture the cells.

Hydrogen peroxide treatment of induced motor neurons. At 14-days post-transduction, induced motor neurons were treated with 100 μM hydrogen peroxide in N3 media for 3 hrs. Cell survival was determined 48 hrs after the removal of hydrogen peroxide and was counted as the fraction of *Hb9*::GFP^{+} neurons remaining after treatment out of the total number of *Hb9*::GFP^{+} neurons before treatment.

Patch clamping. Patch clamp experiments were performed as in Son et al 2012. Intracellular solution was a potassium-based solution and contained KCl, 135mM; $MgCl_2$, 2mM; HEPES, 10mM; MgATP, 4mM; NaGTP, 0.3mM; Na_2PhosCr, 10mM; EGTA, 1mM; pH 7.4. The extracellular was sodium-based and contained NaCl, 135mM; KCl, 5mM; $CaCl_2$, 2mM; $MgCl_2$, 1mM; glucose, 10mM; HEPES, 10mM; pH 7.4. Whole-cell voltage-clamp and current-clamp recordings were made using a Multiclamp 700B (Molecular Devices) at room temperature (21-23°C). Data were digitized with a Digidata 1440A A/D interface and recorded using pCLAMP 10 software (Molecular Devices). Patch pipettes were pulled from borosilicate glass capillaries on a Sutter Instruments P-97 puller and had resistances of 2-4 MΩ. Series resistance was typically 5-10 MΩ, always less than 15 MΩ, and compensated by at least 80%. For study of voltage-gated conductances, voltage was stepped from a holding potential of -80 mV to test potentials from -80 to 30 mV in 10 mV increments. Based on the chloride Nernst potential of -2 mV, inward currents were expected following GABA and glycine treatment[11].

Preparation of cell extracts. For assaying biochemical activities using MEFs, we prepared nuclear extracts as described[12]. Briefly, cells from at least two confluent 15-cm dishes were trypsinized, the cell pellet was washed once with PBS and spun for 2 min at 1200 rpm. The pellet was resuspended in 1 mL 1X PBS and transferred to a 1.5 mL tube. The tubes were centrifuged for 5 min at 1200 rpm. The pellet was resuspended in one volume Buffer A (10 mM HEPES-KOH pH 8.0, 1 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) and incubated for 15 min on ice. A 1-mL syringe (fitted with a 0.5 mm x 16 mm needle) was filled with Buffer A and thereafter fully displaced by the plunger to remove all the remaining air within the syringe. The cells were lysed by slowly drawing the suspension into the syringe followed by rapidly ejecting against the tube wall. This step was repeated five times for complete lysis to occur. The sample was then spun for 20 sec at 13000 rpm and the postnuclear supernatant was discarded. The pellet was resuspended in two-thirds of one packed cell volume in Buffer C (20 mM HEPES-KOH pH 8.0, 1.5 mM $MgCl_2$, 25 % (v/v) glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.1 mM PMSF, 1 mM DTT) and incubated on ice with stirring for 30 min. The suspension was centrifuged for 5 min at 12000 rpm. For RNA kinase, tRNA splicing and inter-strand ligation assays, the supernatant (corresponding to nuclear extract) was dialyzed for 1 hr against 30 mM HEPES-KOH pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 10 % (v/v) glycerol, 1 mM DTT, 0.1 mM AEBSF using dialysis membranes (Millipore 'V' series membrane). For mRNA 3' end cleavage assays, nuclear extracts were instead dialyzed against 30 mM Tris-HCl pH 8.0, 80 mM KCl, 2 mM $MgCl_2$, 10 % (v/v) glycerol, 2 mM DTT, 0.1 mM AEBSF serine protease inhibitor (Sigma). Afterwards, protein concentrations were determined (BioRad Bradford reagent), normalized using dialysis buffer and immediately used for enzymatic assays or snap-frozen and stored at -80°C. In case HeLa cells were used for enzymatic assays, extracts were prepared by lysing cells in 100 mM KCl, 30 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 1 % (v/v) NP-40, 10 % (v/v) glycerol, 0.5 mM DTT, 0.1 mM AEBSF.

Cloning and cell transfection of expression constructs. To generate vectors for the expression of Tyr191 and Tyr958-5' leader-exon tRNA fragments in the NSC-34 cell line, the following DNA oligonucleotides were 5' end-phosphorylated and annealed: Tyr191_Fw 5'-GAT CCC CGT GCT TCC CTT CGA TAG CTC AGC TGG TAG AGC GGA GGA CTG TAG TTT

TTA-3' and Tyr191_Rv 5'-AGC TTA AAA ACT ACA GTC CTC CGC TCT ACC AGC TGA GCT ATC GAA GGG AAG CAC GGG-3'; Tyr958_Fw 5'-GAT CCC CGT GGA TCC TTC GAT AGC TCA GTT GGT AGA GCG GAG GAC TGT AGT TTT TA-3' and Tyr958_Rv 5'-AGC TTA AAA ACT ACA GTC CTC CGC TCT ACC AAC TGA GCT ATC GAA GGA TCC ACG GG-3'. DNA duplexes were subsequently cloned into the pSUPER.neo+GFP vector (Oligoengine) that was pre-cleaved with BglII and HindIII, resulting in a vector from which the tyrosine tRNA fragments were expressed under the control of the H1 promoter. Stable transfections of pSUPER.neo+GFP vectors containing the corresponding tRNA fragment encoding oligonucleotides, or a control vector lacking the insert were performed using Fugene HD transfection agent (Roche) according to the manufacturer's instructions. After 24 hours, selection was started with 500 μg/mL of the neomycin analog G418 (Invitrogen). G418-selected cells were constantly maintained in the presence of the selective drug. GFP expressing cells were sorted by FACS AriaIII. Cloning of the gcDNA3.1 expression vectors (gift from Hartmut Beug, IMP, Vienna) encoding for c-myc-tagged versions of the human CLP1 and CLP1 K127A gene using the Gateway system (Invitrogen) has been previously described[13]. Transfection of expression vectors into MEFs (two 15-cm plates per construct) and HeLa cells (one 6-well plate per construct) was performed using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. Cells were lysed 48 hrs after transfection, and extracts were subjected to enzymatic assays.

Generation of stable HeLa cell lines expressing TAP (Tandem affinity purification)-myc-tagged proteins and TAP purification. Genes of interest were introduced into the pRV-NTAP vector (a gift from Tilmann Buerckstuemmer and Giulio Superti-Furga, CeMM, Vienna) as a destination vector using the Gateway cloning system (Invitrogen). The TAP-tag comprises protein G, followed by two TEV protease cleavages sites, streptavidin binding protein, and a myc-epitope. HEK GP-293 was used as packaging cell line. Successfully infected GFP-positive HeLa target cells containing the insert (TAP constructs express GFP via an internal ribosome entry site) were sorted by FACS and expanded. For Tandem Affinity Purification, cells of five confluent 15-cm dishes stably over-expressing the TAP-tagged protein of interest were trypsinized, cell suspensions were pooled and pelleted for 3 min at 1200 rpm. Cell pellets were then resuspended in 0.75 mL lysis buffer (50 mM Tris-HCl pH 7.5, 5% glycerol, 0.2% NP-40,

1.5 mM $MgCl_2$, 100 mM NaCl, Complete Protease Inhibitor (Roche; 1 tablet per 50 mL buffer, 1 mM DTT) and transferred to a 1.5 mL tube. The samples were incubated on ice for 10 min and then centrifuged for 15 min at 15000 x g. The supernatant was again centrifuged for 20 min at 14000 rpm. 40 µL (settled volume) IgG Sepharose 6 Fast Flow beads (GE Healthcare) were equilibrated in lysis buffer and the cleared cell lysate was added to the beads and incubated for 2 hrs rotating at 13 rpm and 4°C. The beads were collected by centrifugation at 600 rpm and 4°C for 3 min and the supernatant was discarded. The beads were then transferred to a Pierce Spin Column Snap Cap (Thermo Scientific) and washed twice with 500 µL lysis buffer by spinning for 1 min at 2000 rpm. The beads were washed twice with 500 µL TEV cleavage buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA) plus 0.2% (v/v) NP-40. 180 µL TEV cleavage buffer including 20 U AcTEV Protease (Invitrogen) were added to the beads and the column was incubated for 2 hrs at 16°C shaking at 800 rpm. Finally, the column was spun for 1 min at 2000 rpm to collect the eluate, to which glycerol was added to a final concentration of 10% (v/v). TEV eluates were then directly used for enzymatic assays or snap frozen and stored at -80°C.

Retroviral infection of MEFs. pMX-IRES-GFP empty vector and pMX-Flag-Clp1-IRES-GFP were transfected into Plat-E cells[14] using Fugene 6 transfection reagent (Roche). Cells were incubated for 48 hrs at 37°C with 5% CO_2. MEFs were seeded at 2×10^5 cells per 6 well. The supernatants derived from these Plat-E cultures were centrifuged at 1500 rpm for 5 min and supplemented with 4 µg/mL polybrene (Sigma). MEFs were incubated in the virus/polybrene-containing supernatants for 8 hrs, then 1 mL of fresh medium was added After 24 hrs of infection, the cells were replated in 10 mL fresh medium.

RNA kinase assay and inter-strand ligation assay. 50 pmol RNA oligonucleotide derived from the firefly luciferase gene (5'-UCG AAG UAU UCC GCG UAC GU-3', Dharmacon) were incubated with 1.11 MBq [5'-^{32}P] cytidine-3',5'-bisphosphate (111 TBq/mmol, Perkin Elmer) and 20 units T4 RNA ligase 1 (NEB) for 1 hr at 16°C in 15% (v/v) DMSO, 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 200 µM ATP, 0.1 mg/mL BSA in a total reaction volume of 10 µL. Labeling reactions were resolved by denaturing gel electrophoresis in 15% polyacrylamide gels containing 8 M urea (SequaGel, National

Diagnostics). The 3' end-labeled RNA was visualized by autoradiography and passively eluted from gel slices overnight in 0.3 M NaCl. The RNA was precipitated by adding three volumes of ethanol and for the interstrand-ligation assay directly annealed to a non-labeled complementary RNA oligonucleotide (5'-CGU ACG CGG AAU ACU UCG A-3', Dharmacon) as described below. Alternatively, for the RNA kinase assay the 3' end-labeled RNA was first dephosphorylated (in a 120 µL reaction, 1 U Alkaline Phosphatase AP (Roche) and Roche-supplied buffer) for 30 min at 37°C. The reaction was then de-proteinized by Proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The RNA was further annealed with a non-labeled complementary RNA oligonucleotide (5'-CGU ACG CGG AAU ACU UCG AAA-3', Dharmacon). Annealing reactions were performed as follows: 50 nM labeled and non-labeled complementary RNA oligonucleotide were mixed in 30 mM HEPES-KOH pH 7.5, 2 mM $MgCl_2$ and 100 mM KCl, heated to 95°C for 2 min and subsequently incubated at 37°C for 1 hr. The obtained RNA duplexes were directly used as substrates for the inter-strand ligation or RNA kinase assay.

The RNA kinase activity assay was performed by adding equal volumes of cell extracts and reaction mixture (100 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, 0.4 mM GTP and 64 U/ml RNasin [Promega]) containing 10 nM radio-labeled RNA duplex, followed by incubation at 30°C. At given time points, 5 µL of the reaction was stopped by adding 5 µL of 8 M urea solution. Reaction products were separated on a 15 % denaturing acrylamide gel containing 8 M urea (Sequagel, National Diagnostics), and RNA phosphorylation was monitored by phosphorimaging. To test for inter-strand ligation, 3 µL reaction mixture (167 µM EDTA pH 8.0, 67 mM KCl, 2 mM $MgCl_2$, 8.3 mM DTT, 5 mM ATP, 0.3 mM GTP, 53 U/mL RNasin (Promega), 43% glycerol) containing 17 nM radio-labeled RNA duplex were mixed with 2 µL cell extract and incubated for 30 min at 30°C. Reactions were de-proteinized with Proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. Reaction products were separated on a 15% denaturing polyacrylamide gel containing 8 M urea (Sequagel, National Diagnostics) and radiolabeled RNA was visualized by phosphorimaging.

Pre-tRNA cleavage and splicing assays. A PCR was performed using *S. cerevisiae* genomic DNA as template, a 5' primer including the T7 polymerase promoter (5'-AAT TTA ATA CGA

CTC ACT ATA GGG GAT TTA GCT CAG TTG GG- 3'), and a 3' primer (5'-TGG TGG GAA TTC TGT GGA TCG AAC-3'). The PCR product was sequenced and identified as yeast tRNA3-Phe^{GAA} (chromosome 13). The PCR product served as template for *in vitro* transcription using the T7 MEGAshortscript kit (Ambion) including 1.11 MBq [α-^{32}P]guanosine-5'-triphosphate (111 TBq/mmol, Perkin Elmer) per reaction. The pre-tRNA was resolved in a 10 % denaturing polyacrylamide gel containing 8 M urea (Sequagel, National Diagnostics), visualized by autoradiography and passively eluted from gel slices overnight in 0.3 M NaCl. RNA was precipitated by addition of three volumes of ethanol, and dissolved at 1 μM in buffer containing 30 mM HEPES-KOH pH 7.3, 2 mM $MgCl_2$, 100 mM KCl. To assess pre-tRNA cleavage or splicing, four volumes of reaction buffer (100 mM KCl, 5.75 mM $MgCl_2$, 2.5 mM DTT, 5 mM ATP, 6.1 mM Spermidine-HCl pH 8.0 (Sigma) and one volume of 1 μM body-labeled *S. cerevisiae* pre-tRNA^{Phe} were mixed, heated at 95°C for 90 sec and incubated for 20 min at room temperature. RNasin RNase inhibitor (Promega) was added to a final concentration of 6 U/mL. Equal volumes of the described reaction mixture and cell extracts or TEV eluates were mixed and incubated at 30°C. At given time points, 5 μL of the mix were deproteinized with Proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. Reaction products were separated on a 10% denaturing polyacrylamide gel containing 8 M urea (Sequagel, National Diagnostics), and mature tRNA formation and/or tRNA exon formation was monitored by phosphorimaging. Quantification of band intensities was performed using the ImageQuant TL 7.0 software (Amersham).

mRNA 3' end cleavage assay. Equal volumes of MEF nuclear extracts and reaction buffer (5.2% polyvinyl alcohol, 80 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 40 mM creatine phosphate, 0.02 μg/μL creatine kinase, 1 mM cordycepin 5′-triphosphate, 1 mM EDTA) including 20 nM capped ^{32}P-labeled SV40 late pre-mRNA (*in vitro* transcribed using pSV-L (SV40) as template; gift from Walter Keller, University of Basel) were mixed and incubated at 30°C. At given time points, 5 μL of the mix were deproteinized with Proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. Reaction products were analyzed by 6% denaturing polyacrylamide gel electrophoresis (Sequagel, National Diagnostics) and radiolabeled RNA was visualized by phosphorimaging.

Northern blot analysis. Isolation of total RNA from tissues and cultured cells was performed using the Trizol Reagent (Invitrogen) or RNeasy (Qiagen). For isolating RNA after subcellular fractionation, we essentially followed the protocol described in "Preparation of cell extracts" and used the Trizol reagent to recover RNA from the postnuclear supernatant ("cytoplasm") and the nuclear pellet ("nucleus") after the centrifugation step for 20 sec at 13000 rpm. 5 µg of RNA (or RNA from cytoplasmic and nuclear fraction derived from the same number of cells) was separated in a 10% denaturing polyacrylamide gel (20 x 25 cm) (Sequagel, National Diagnostics). The RNA was blotted on Hybond-N+ membranes (GE Healthcare) and fixed by ultraviolet cross-linking. Membranes were baked for 1 hr at 80°C and pre-hybridized in 5X SSC, 20 mM Na_2HPO_4 pH 7.2, 7 % SDS, 1x Denhardt's solution and 0.1 mg/mL sonicated salmon sperm DNA (Stratagene) for 1 hr at 80°C. Hybridization was performed in the same buffer overnight at 80°C (for DNA/LNA probes) or 50°C (for DNA probes) including 100 pmol of the following [5'-^{32}P]labeled DNA/LNA probes (Exiqon, Denmark; LNA nucleotides are indicated by "*X"): tyrosine tRNA 5' exon probe, 5'-CT*A CA*G TC*C TC*C GC*T CT*A CC-3'; tyrosine tRNA 3' exon probe, 5'-CG*A AC*C AG*C GA*C CT*A AG*G AT-3', or following DNA probes: tyrosine tRNA 5' exon probe, 5'-CTA CAG TCC TCC GCT CTA CCA-3'; arginine tRNA 5' exon probe, 5'-TAG AAG TCC AAT GCG CTA TCC-3'; isoleucine tRNA 5' exon probe, 5'-TAT AAG TAC CGC GCG CTA ACC-3'; leucine tRNA 5' exon probe, 5'-CTT GAG TCT GGC GCC TTA GAC-3'; methionine 5' tiRNA probe, 5'-GGG CCC AGC ACG CTT CCG CTG CGC CAC TCT GC-3'; proline 5' tiRNA probe, 5'-CAA AGC GAG AAT CAT ACC CCT AGA CCA ACG AGC C-3'. Subsequently blots were washed twice for 1 min at 50°C with 5x SSC, 5% SDS and once for 1 min with 1x SSC, 1% SDS and analyzed by phosphorimaging. Membranes were routinely re-hybridized at 50°C using a DNA probe (5'-GCA GGG GCC ATG CTA ATC TTC TCT GTA TCG-3') complementary to U6 snRNA to check for equal loading of RNA amounts.

TSEN2 siRNA-mediated knock-down experiments. MEFs at 30% confluency were transfected in a 6-cm tissue culture plate with 100 nM siRNA duplexes targeting TSEN2 (Dharmacon, ON-TARGETplus SMARTpool, catalog number L-050573-01-0005, pool of four siRNAs targeting following mRNA sequence of mouse TSEN2: 5'-GGA AGG GUU ACU UUG GAA A-3', 5'-UGA GCA GAG UCU CGG GGA A-3', 5'-CAU GCA AGC UAC UCG

GUC A-3', 5'-CUA UGA GAG UUA CGA GUC A-3') or with custom-made control siRNAs (Dharmacon) directed against the firefly luciferase gene (mRNA target sequence 5'- CGU ACG CG G AAU ACU UCG AAA-3') using Lipofectamine 2000 according to the manufacturer's instructions, or were left untransfected. After 48 hours cells were split onto a 10-cm dish and re-transfected with siRNAs at 100 nM on the next day. Total RNA was isolated after another 72 hours using the Trizol reagent (Invitrogen) and analyzed by Northern blotting. TSEN2 knock-down efficiency was confirmed by quantitative real-time PCR.

Quantitative real-time PCR. First-strand cDNA synthesis was carried out using the High Capacity RNA-to-cDNA Master Mix kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR was performed using GoTaq qPCR Master Mix kit (Promega) and the Bio-Rad iQ5 qPCR device. Sense and antisense primers, respectively, were as follows: TSEN2, 5' -TCC GTG CTC CTA AGA GGA AA-3' and 5'-GGT TGG CGA TTG TGA AGT TT-3'; GAPDH, 5'-CGT CCC GTA GAC AAA ATG GT-3' and 5'-GAA TTT GCC GTG AGT GGA GT-3'. The PCR was performed in 25 μL volumes using following parameters: 50°C for 10 min, 95°C for 5 min, followed by 60 cycles in total at 95°C for 10 sec and 60°C for 30 sec. All experiments were performed in triplicate, and the generation of specific PCR products was confirmed by melting curve analysis and DNA gel electrophoreses. The data were analyzed according to the ΔΔCt method normalizing to GAPDH mRNA levels.

Exon microarray data and differential splicing analysis. RNA extracted from spinal cord of three wild type and three *Clp1*$^{K/K}$ mice (male, 15 days-old) was depleted from ribosomal RNA and subjected to alternative splicing analysis employing Affymetrix Mouse Exon microarrays (MoEx 1.0 ST v1). Raw data pre-processing, custom probe alignments and differential splicing analysis was performed similar to Ref. 15. In brief, probe sequences of the Mouse Exon microarray were aligned against the mouse genome and probes with complete alignments were annotated to exons/genes if they aligned within the exon boundaries of the respective gene. Gene/exon definitions were taken from the Ensembl core database (version 52) and the Alternative Spliced Transcript Database (version 1.1)[16]. A custom CDF file was compiled defining gene probe sets, i.e. collections of probes targeting any exon of a specific gene. Raw signals from all microarrays were background adjusted using the GCRMA method[17] and

quantiles normalized. Probe residuals from fitting the RMA[18] model to the probe signals of a gene probe set were used to estimate alternative splicing. These probe residuals represent deviations of a probe's signal from the expected, averaged gene expression value within the same sample and are thus an indicator for alternative inclusion/exclusion of a complete or a part of an exon. Further analysis was restricted to 50% of the genes with the highest probe residual variances. The residuals were compared for each probe between *Clp1*$^{K/K}$ and wild type samples employing the moderated t-test[19]. Resulting p-values were adjusted for multiple hypothesis testing using the method of Benjamini and Hochberg[20]; adjusted p-values <0.05 were considered significant, thus accepting a 5% false discovery rate among the rejected hypothesis. Raw and pre-processed data have been deposited at the Gene Expression Omnibus (GEO). All primary data can be accessed using the link (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=znqpxiukwocyefi&acc=GSE35924). The tRNA fragment analysis has the accession number GSE39275.

Dual luciferase reporter assays. MEFs were transfected in 24-well plates with 10 ng pGL3 vector (Promega) encoding for firefly luciferase together with 190 ng of pRL-CMV (control) or pRL-3xBulgeA (a gift from Witek Filipowicz, FMI Basel), containing three bulged binding sites for let-7a miRNA at the 3′-UTR of the *Renilla* luciferase gene. Transfection was performed in triplicates using Lipofectamin 2000. Luciferase activity was assayed 40 hours after transfection using the Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. *Renilla* luciferase activity was normalized according to firefly luciferase activity.

Cloning, RNA sequencing and bioinformatics analysis on tRNA fragments. ~50 µg of total RNA from primary MEFs was separated alongside with a radiolabeled RNA decade marker (Ambion) on a preparative 10% denaturing polyacrylamide gel (20 × 25 cm, 1 mm thick) containing 8 M urea (Sequagel, National Diagnostics). The gel region comprising sizes of 37-50 nucleotides was excised and passively eluted overnight into 0.4 M NaCl. The RNA was precipitated by adding three volumes of ethanol, the pellet was washed once in 85 % ethanol and dissolved in 16 µL H_2O. 2 µL (10 U/µL) tobacco acid pyrophosphatase (TAP, Epicentre; in order to hydrolyze the terminal 5' triphosphate group of tRNAs to generate a 5' monophosphate

terminus; alternatively RNA 5' polyphosphatase (Epicentre) was used to produce 5' monophosphate termini) and 2 μL 10x TAP reaction buffer was added followed by incubation for 1 hour at 37°C. The reaction was deproteinized, and the RNA was extracted with phenol/chloroform and subsequently precipitated with ethanol. Adaptor ligation for high-throughput sequencing was essentially performed as described in Ref. 21 except that the 5' adapter ligation step was performed prior to the 3' adapter ligation. 5' adapter oligonucleotides (5'-GUU CAG AGU UCU ACA GUC CGA CGA UC-3') were ligated using T4 RNA ligase I (NEB) including 15 % polyethylene glycol 8000 on ice overnight. After separation by a preparative 10% denaturing polyacrylamide gel, the gel region corresponding to 60-74 nucleotides was excised, the RNA passively eluted and ethanol precipitated as described above. The RNA was ligated to 3' adapter oligonucleotides (5'- App-UCG UAU GCC GUC UUC UGC UUG UidT-3') using T4 RNA ligase 2 (truncated, NEB) including 15% polyethylene glycol 8000 on ice overnight. RNA was again separated on a 10% denaturing polyacrylamide gel, and RNA was eluted from the gel region corresponding to 80-100 nucleotides. For cDNA preparation, Illumina Reverse primer (5'-CAA GCA GAA GAC GGC ATA CGA-3') and Superscript II Reverse Transcriptase kit (Invitrogen) was used. PCR amplification was performed using Phusion High Fidelity Polymerase (NEB) and Illumina Forward (5'- AAT GAT ACG GCG ACC ACC GAC AGG TTC AGA GTT CTA CAG TCC GA-3') and Reverse primers. PCR products were subsequently sequenced on an Illumina G2 platform. The reads were trimmed with cutadapt (v 1.0)[22] to remove adaptors. Trimmed reads longer than 15-nts were aligned with bowtie (v 0.12.5)[23] to the mouse genome (ncbi build 37) allowing 2 mismatches. The uniquely aligned reads were classified by overlap with tRNA locations (Lowe lab, http://gtrnadb.ucsc.edu/Mmusc/Mmusc-by-locus-txt.html) using bedtools (v 2.16.2) tagBam[24].

Identification of the 5' end modification of the tyrosine tRNA fragment. Total RNA from primary *Clp1*$^{K/K}$ MEFs was separated alongside with a radiolabeled RNA decade marker (Ambion) on a preparative 10% denaturing polyacrylamide gel (20 × 25 cm, 1 mm thick) containing 8 M urea (Sequagel, National Diagnostics). The gel region comprising sizes of 37-50 nucleotides was excised and passively eluted overnight into 0.4 M NaCl. The RNA was precipitated by adding three volumes of ethanol, the pellet was washed once in 85% ethanol and

dissolved in H_2O. Half of the RNA was treated with RNA 5' polyphosphatase (Epicentre) for 1 hr at 37°C according to the manufacturer's instructions, the other half was left untreated. RNA was subsequently deproteinized, extracted with phenol/chloroform, precipitated with ethanol and dissolved in H_2O. One third of the RNA solution was left untreated, one third denatured at 50°C for 5 min and treated with recombinant Xrn1 (NEB) for 1 hr at 37°C, and one third was ligated with 5' Illumina adapter oligonucleotides (5'-GUU CAG AGU UCU ACA GUC CGA CGA UC-3') using T4 RNA ligase I (NEB) including 15% polyethylene glycol 8000 on ice overnight. Reaction products were subsequently subjected to Northern blotting and detected with a DNA/LNA probe complementary to the tyrosine tRNA 5' exon.

Statistics. All values in the paper are given as means ± S.D. Comparisons between groups were made by Student's t-test and one-way ANOVA. For the Kaplan Meier analysis of survival, a log rank test was performed. $P < 0.05$ was accepted as statistically significant.

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