Supplemental material for:

New *Lmna* knock-in mice provide a molecular mechanism for the "segmental aging" in Hutchinson-Gilford progeria syndrome

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SUPPLEMENTAL METHODS

Luciferase reporter assay

Luciferase reporter vectors (pmirGLO, Promega) with full-length prelamin A 3′ UTR, mutant versions of prelamin A's 3′ UTR with 3-nucleotide mutations (CAA>ACC, CAA>del) (Supplementary Material, Fig. S1A), or lamin C's 3′ UTR were reported previously (1). A luciferase reporter vector with the "ACCCT" mutation was generated by site-directed mutagenesis with primer 5′–AGCAGGCCTGAAGACCCTGAAAAATTTATC–3′ and a complementary reverse primer. HeLa and U-87 MG cells were cotransfected with the luciferase vectors along with either a miR-9 expression vector or an empty vector (2). After 24 h, the cells were lysed with Passive Lysis Buffer (Promega) and analyzed for firefly- and Renilla-luciferase activities with the Dual-Luciferase Reporter Assay System (Promega) and a Synergy 2 luminometer (Biotek).

Myeloid cell studies

Bone marrow cells were harvested from the femur and tibia by flushing with PBS. Red blood cells were removed by incubation in red blood cell lysis buffer (0.15 M NH_4Cl, 10

mM $KHCO_3$, 0.1 mM EDTA) at 37º C for 3 min. Peritoneal cells were collected by peritoneal lavage with PBS and resident macrophages were isolated by cell adherence in DMEM medium containing 10% FBS at 37ºC for 1 h. After washing with PBS twice, adherent cells were collected with cell scrapers.

For protein analysis, cells were lysed in urea buffer and processed as described in the *Materials and Methods.* microRNA levels were measured by qRT-PCR as described (1). Total RNA was isolated with an RNeasy kit (Qiagen) and reverse-transcribed with miRCURY LNA Universal cDNA synthesis kit II (Exiqon). qPCR reactions were performed on a 7900 Fast Real-Time PCR system (Applied Biosystems) with the ExiLENT SYBR Green PCR Master Mix and LNA PCR primers (Exiqon).

Fluorescence *in situ* hybridization

One-month-old mice were perfused with 4% paraformaldehyde (PFA) and tissues were cryoprotected in 20% sucrose overnight at 4º C. Tissues were then embedded in Optimum Cutting Temperature compound (Sakura Finetek) and sectioned (10 µm). *In situ* hybridization was performed with the miRCURY LNA microRNA ISH Optimization kit (Exiqon). Slides were treated with proteinase K (2 mg/ml) for 10 min at 37º C and then incubated in 3% hydrogen peroxidase in PBS to block endogenous peroxidase activity. After dehydration in 70%, 96%, and 99.9% ethanol, the slides were incubated with digoxigenin-labeled probes (40 nM) in 1× microRNA ISH buffer (Exiqon) for 1 h at 55º C and washed with 5×, 1×, and 0.2× SSC buffers (5 min each) at 55º C. Slides were then incubated with polymerized horseradish peroxidase (POD)–conjugated anti-digoxigenin sheep IgG (Roche). Antibody binding was detected with the TSA Plus Systems (PerkinElmer).

REFERENCES

1 Jung, H.J., Coffinier, C., Choe, Y., Beigneux, A.P., Davies, B.S., Yang, S.H., Barnes, R.H., 2nd, Hong, J., Sun, T., Pleasure, S.J. *et al.* (2012) Regulation of prelamin A but not lamin C by miR-9, a brain-specific microRNA. *Proc*. *Natl*. *Acad*. *Sci*. *U*. *S*. *A*., **109**, E423-431.

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4 Jung, H.J., Nobumori, C., Goulbourne, C.N., Tu, Y., Lee, J.M., Tatar, A., Wu, D., Yoshinaga, Y., de Jong, P.J., Coffinier, C. *et al.* (2013) Farnesylation of lamin B1 is important for retention of nuclear chromatin during neuronal migration. *Proc*. *Natl*. *Acad*. *Sci*. *U*. *S*. *A*, **110**, E1923-1932.

SUPPLEMENTAL FIGURES

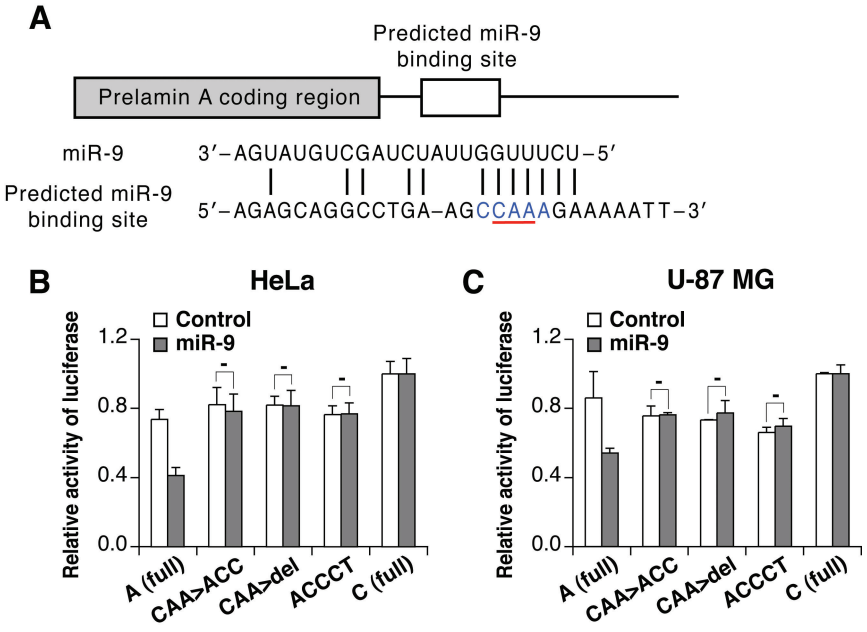

Figure S1. The "ACCCT mutation" in the seed-binding sequence of the miR-9 binding site in prelamin A's 3′ UTR abolishes the effects of miR-9 on prelamin A expression. (A) Mutation of the predicted miR-9 binding site in prelamin A's 3′ UTR. The location of the CCAAAG => ACCCTG mutation is indicated by blue font; the location of the three-nucleotide mutations (CAA>ACC and CAA>del), which were described previously (1), are underlined in red. (B and C) Luciferase assays in HeLa cells (B) and U-87 MG cells (C) that had been transfected with an empty vector (control) or a miR-9 expression vector (miR-9) along with luciferase reporter vectors in which the miR-9 seed-binding sequence (CCAAAG) in prelamin A's 3′ UTR was mutated. From left to right: A (full), wild-type 3′ UTR of prelamin A; CAA>ACC, substitution of nucleotides CAA in prelamin A's 3′ UTR with ACC; CAA>del, deletion of the CAA in prelamin A's 3′ UTR; ACCCT, substitution of the five nucleotides in prelamin A's 3′ UTR (CCAAA) with ACCCT; C (full), wild-type 3′ UTR of lamin C (1). Firefly luciferase activity levels were normalized to Renilla luciferase activity levels and compared to those in cells transfected with the luciferase vector containing the wild-type 3′ UTR of lamin C (set at 1.0). Values represent mean ± SD. The ACCCT mutation abolished the ability of miR-9 to reduce luciferase expression in both HeLa cells and U-87 MG cells. Consistent with earlier studies in HeLa cells (1), mutating (or deleting) three nucleotides (CAA) in the predicted miR-9 binding site also blocked the inhibition by miR-9. In these cell culture studies, the levels of miR-9 expression in the transfected cells were only ~10% of the levels normally observed in the cerebral cortex of wild-type mice (1). (–), $p > 0.05$.

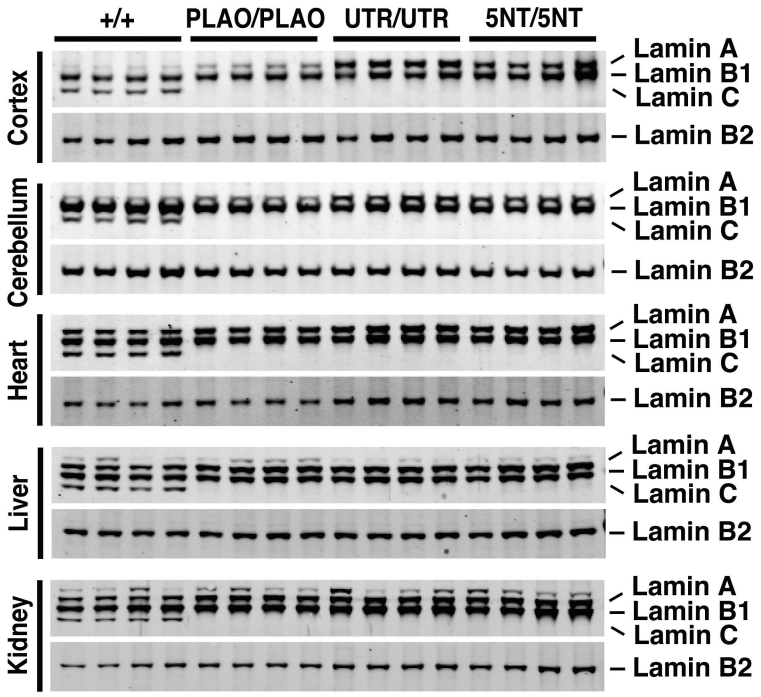

Figure S2. Western blot analysis of lamin B1 and lamin B2 expression in the cerebral cortex, cerebellum, heart, liver, and kidney from *Lmna*$^{+/+}$ (+/+), *Lmna*$^{PLAO/PLAO}$ (PLAO/PLAO), *Lmna*$^{PLAO\text{-}UTR/PLAO\text{-}UTR}$ (UTR/UTR), and *Lmna*$^{PLAO\text{-}5NT/PLAO\text{-}5NT}$ (5NT/5NT) mice. The same blots shown in Fig. 2A were re-probed with antibodies against lamin B1 and lamin B2. (See Fig. 2B–D for quantification of lamin B1 and lamin B2 signals.)

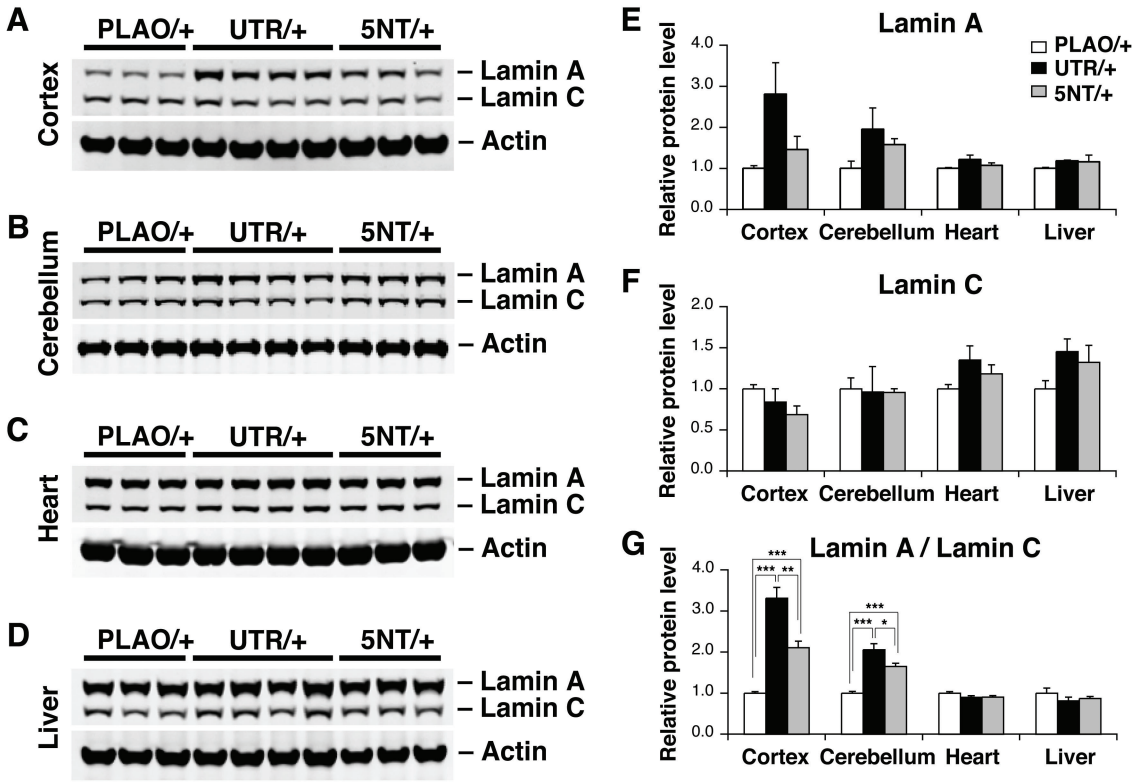

Figure S3. Western blot analysis of tissue extracts from *Lmna*$^{PLAO/+}$ (PLAO/+), *Lmna*$^{PLAO\text{-}UTR/+}$ (UTR/+), and *Lmna*$^{PLAO\text{-}5NT/+}$ (5NT/+) mice with antibodies against lamin A/C and actin. (A–D) Western blots of tissue extracts from 1-month-old mice. (E–G) Quantification of lamin A and lamin C levels relative to actin measured in panels A–D. Values represent mean ± SD. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

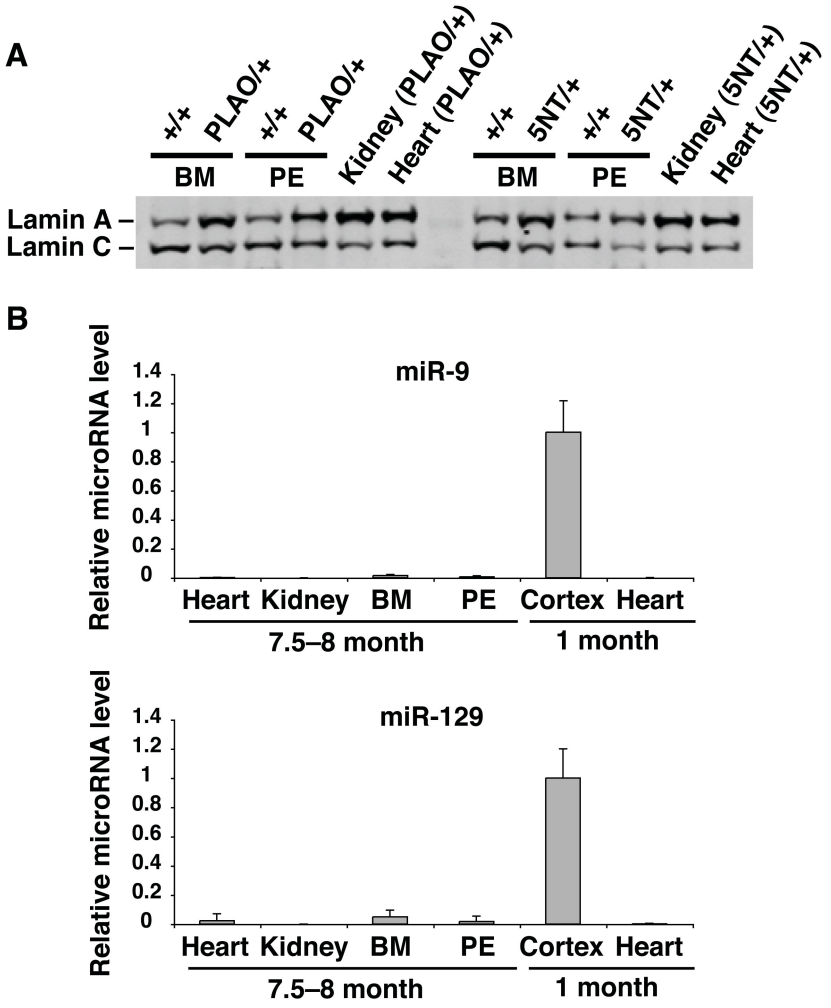

Figure S4. Lamin A and miR-9 expression in myeloid cells. (A) Western blot analysis of protein extracts of bone marrow myeloid cells (BM) and adherent peritoneal macrophages (PE) from *Lmna*$^{PLAO/+}$ (PLAO/+), *Lmna*$^{PLAO\text{-}5NT/+}$ (5NT/+), and wild-type (+/+) littermate mice. (B) qRT-PCR analysis of the levels of miR-9 or miR-129 in myeloid cells. Data was normalized to U6 snRNA. Values represent mean ± SD from four 1-month-old mice and four 7.5–8-month-old mice.

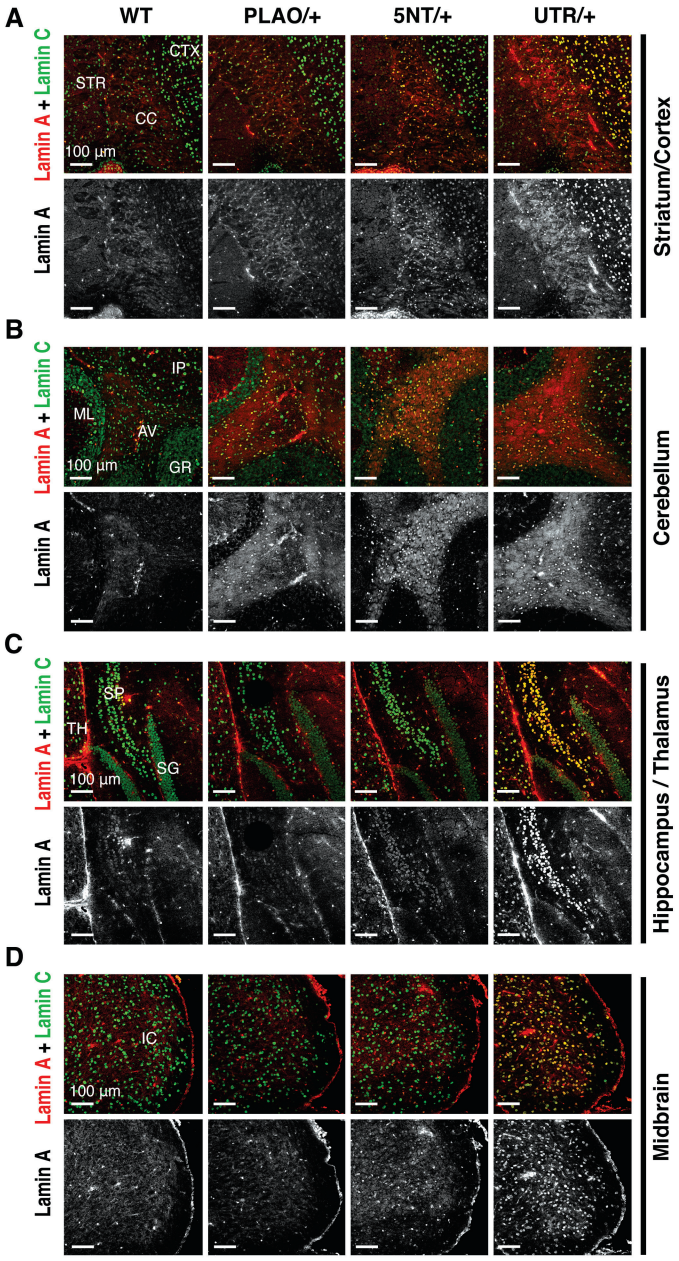

Figure S5. Immunofluorescence microscopy of the striatum/cortex, cerebellum, hippocampus/thalamus, and midbrain from wild-type (WT), *Lmna*$^{PLAO/+}$ (PLAO/+), *Lmna*$^{PLAO\text{-}5NT/+}$ (5NT/+), and *Lmna*$^{PLAO\text{-}UTR/+}$ (UTR/+) mice stained for lamin A (red or white) and lamin C (green). Scale bar, 100 µm. CTX, cerebral cortex; STR, striatum; CC, corpus callosum; ML, molecular layer; AV, arbor vitae; GR, granular layer; IP, interposed nucleus; SG, strata granulosum; SP, strata pyramidale; TH, thalamus; IC, inferior colliculus.

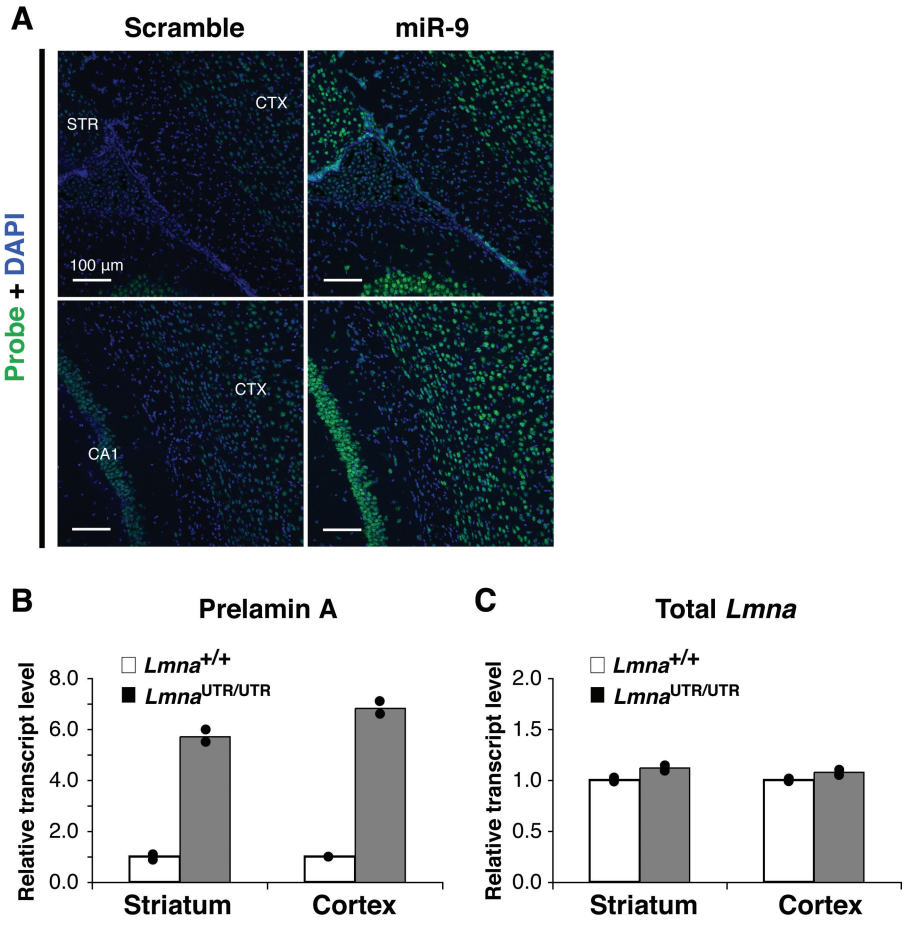

Figure S6. Expression of miR-9 and prelamin A transcripts in the striatum. (A) Fluorescence *in situ* hybridization on brain sections from 1-month-old *Lmna*$^{\text{PLAO-UTR/+}}$ mice with a miR-9 probe or a scrambled control probe. CTX, cerebral cortex; STR, striatum. Scale bar, 100 µm. (B) Quantitative RT-PCR analysis of prelamin A transcript levels and total *Lmna* transcript levels in the striatum and the cerebral cortex of *Lmna*$^{+/+}$ (n = 2) and *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ mice (n = 2). Transcript levels were normalized to cyclophilin A and compared to levels in wild-type mice (set at 1.0).

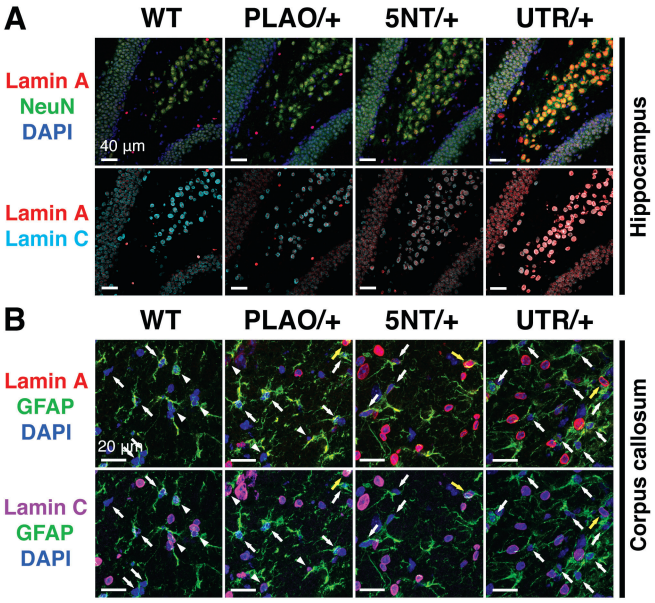

Figure S7. Immunofluorescence microscopy of the hippocampus or the corpus callosum from *Lmna*$^{PLAO/+}$ (PLAO/+), *Lmna*$^{PLAO\text{-}5NT/+}$ (5NT/+) and *Lmna*$^{PLAO\text{-}UTR/+}$ (UTR/+) mice stained with antibodies against lamin A (red), lamin C (cyan in Panel A; magenta in Panel B), and either NeuN (green) in Panel A or GFAP (green) in Panel B. White arrowheads, lamin A–negative/lamin C–positive cells; white arrows, lamin A–negative/lamin C–negative cells; yellow arrows, lamin A–positive/lamin C–positive cells. Scale bars: (A) 40 µm; (B) 20 µm.

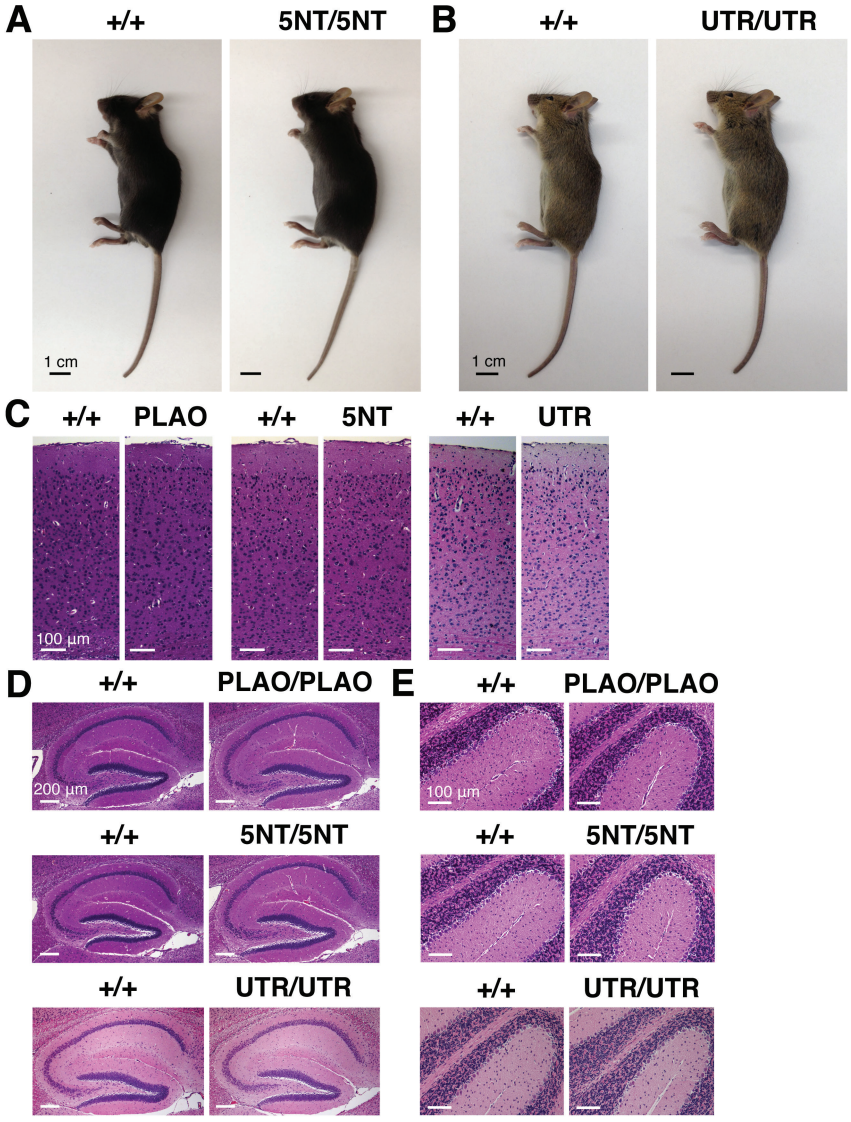

Figure S8. *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ and *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ mice are free of pathology. (A and B) Photographs of 1-month-old *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ (5NT/5NT), *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ (UTR/UTR), and wild-type (+/+) littermate mice. Scale bar, 1 cm. (C–E) Hematoxylin and eosin (H&E)–stained sections of the cerebral cortex (C), hippocampus (D), and cerebellum (E) from 1-month-old *Lmna*$^{\text{PLAO/PLAO}}$ (PLAO or PLAO/PLAO), *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ (5NT or 5NT/5NT) *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ (UTR or UTR/UTR), and wild-type (+/+) littermate mice. Scale bars: panel C, 100 µm; panel D, 200 µm; panel E, 100 µm.

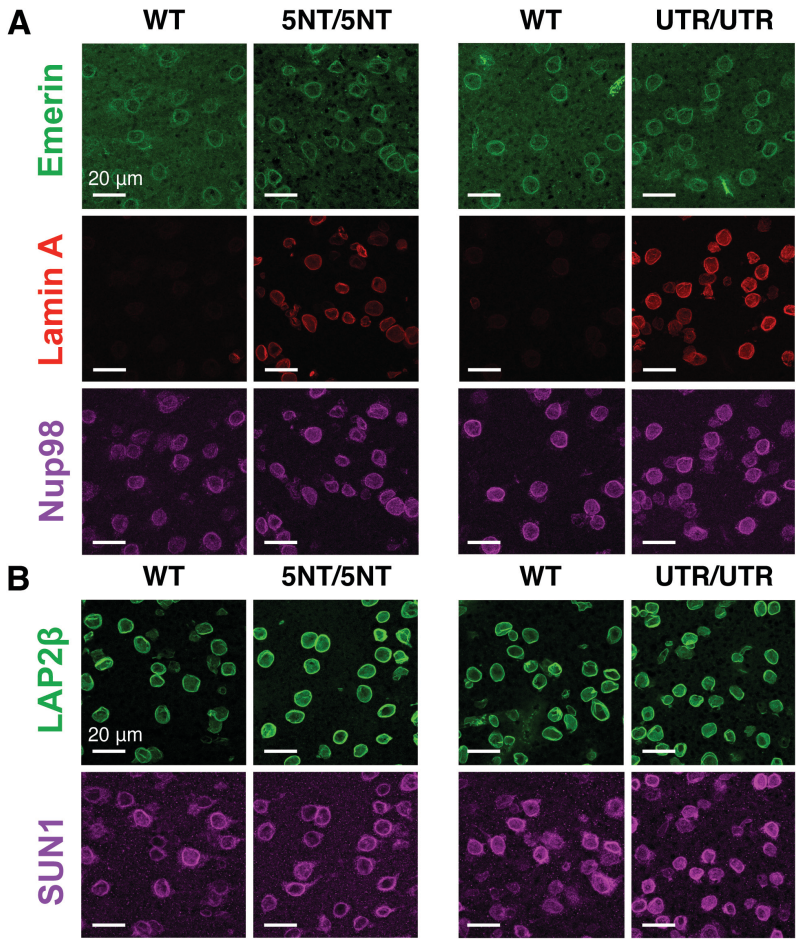

Figure S9. Increased lamin A expression in the brain does not affect localization of other nuclear membrane proteins. The cerebral cortex from *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ (5NT/5NT), *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ (UTR/UTR), and wild-type (WT) littermate mice was stained with antibodies against emerin (green), lamin A (red), and Nup98 (magenta) in (A) and LAP2β (green) and SUN1 (magenta) in (B). Scale bar, 20 μm.

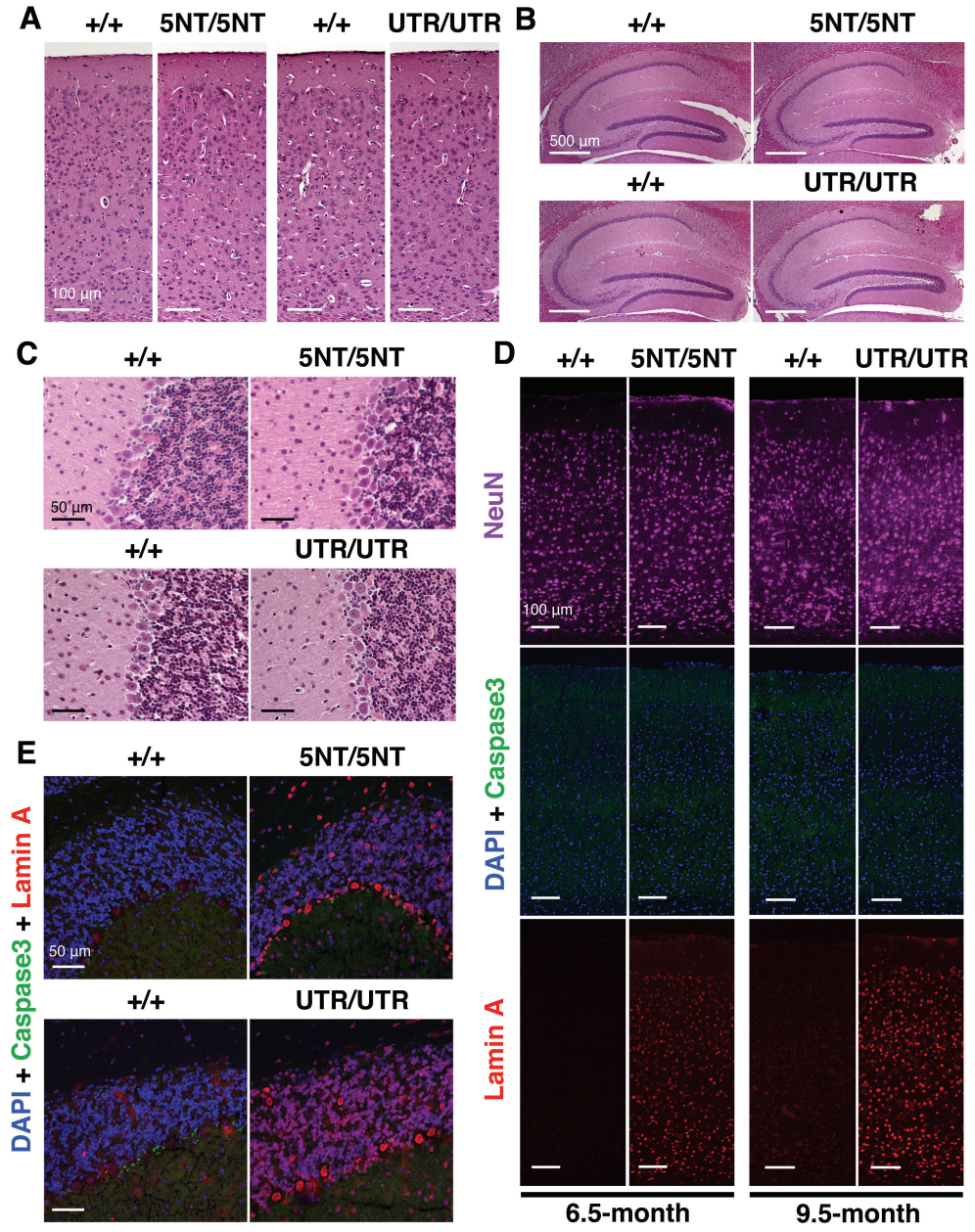

Figure S10. Absence of neuropathology in the brains of older *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ and *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ mice. (A–C) H&E–stained sections of the cerebral cortex (A), hippocampus (B), and cerebellum (C) from 6.5-month-old *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ (5NT/5NT) mice and 9.5-month-old *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ (UTR/UTR) mice along with wild-type (+/+) littermate mice. Scale bars: panel A, 100 µm; panel B, 500 µm; panel C, 50 µm. (D) Immunofluorescence microscopy of the cerebral cortex from 6.5-month-old *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ (5NT/5NT) and 9.5-month-old *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ (UTR/UTR) mice stained with antibodies against NeuN (magenta), active caspase 3 (green), and lamin A (red). Scale bar, 100 µm. (E) High-magnification images of the cerebellum from 6.5-month-old *Lmna*$^{\text{PLAO-5NT/PLAO-5NT}}$ (5NT/5NT) mice, 9.5-month-old *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ (UTR/UTR) mice, and wild-type (+/+) littermate mice stained with active caspase 3 (green) and lamin A (red). Scale bar, 50 µm.

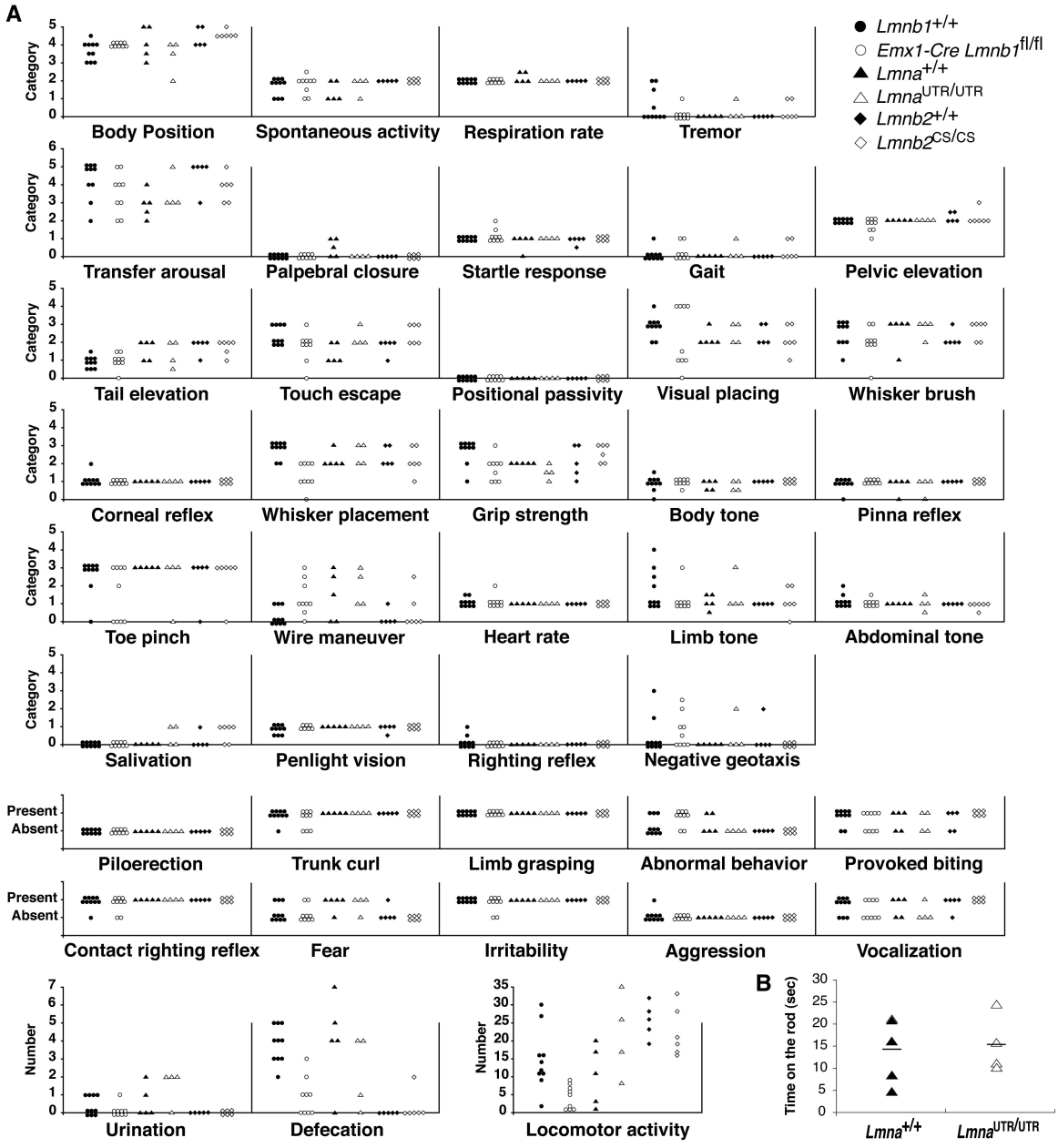

Figure S11. *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ mice are indistinguishable from wild-type mice by SHIRPA behavioral screens (A) and by rotarod tests (B). Forebrain-specific *Lmnb1* knockout mice (*Emx1-Cre Lmnb1*$^{\text{fl/fl}}$) (3), knock-in mice expressing nonfarnesylated lamin B2 (*Lmnb2*$^{\text{CS/CS}}$) (4), and wild-type littermates were included as controls. No statistically significant differences were observed between wild-type mice and *Lmna*$^{\text{PLAO-UTR/PLAO-UTR}}$ mice. See Supplementary Material, Table S2 for the description of each test.

		WT	PLAO	5NT	UTR	Lamin C
Cerebellum	Granule cells	–	–	–	–	+++
	Purkinje cells	–	–	–	++	++++
	Arbor vitae	–	++	++++	++++	+++
	Interposed nucleus	–	–	–	++	++++
	Molecular layer	–	–	–	–	++
Midbrain	Inferior colliculus	–	–	+	+++	++++
Cerebrum	Cerebral cortex	+	++	+++	++++	++++
	Corpus callosum	+	++	++++	++++	+++
	Striatum	–	–	–	+	++
Hippocampus	Strata granulosum	–	–	–	++	+++
	Strata pyramidale	+	+	++	++++	++++
	Thalamus	–	–	–	+++	++++
Olfactory bulb	Granule cells	–	–	–	++	+++
	Glomerular layer	–	–	–	++	+++
	Mitral cells	–	–	–	+	++++

Table S1. Lamin A expression in different regions of the brain from *Lmna*$^{PLAO\text{-}5NT/+}$ and *Lmna*$^{PLAO\text{-}UTR/+}$ mice, as judged by immunohistochemistry. +, ++, +++, and ++++ indicate the intensity of lamin A staining by immunohistochemistry [from + (very weak) to ++++ (very strong)]; – denotes undetectable lamin A expression (Fig. S4). Blue, regions of the brain in which lamin A expression was increased in *Lmna*$^{PLAO/+}$ mice compared with *Lmna*$^{+/+}$ mice; yellow, regions of the brain in which lamin A expression was increased in *Lmna*$^{PLAO\text{-}5NT/+}$ mice compared with *Lmna*$^{PLAO/+}$ mice; green, regions of the brain in which lamin A expression was increased in *Lmna*$^{PLAO\text{-}UTR/+}$ mice compared with *Lmna*$^{PLAO\text{-}5NT/+}$ mice; grey, regions of the brain in which lamin A expression changed very little, even in *Lmna*$^{PLAO\text{-}UTR/+}$ mice.

View Jar	Body Position	0-Completely flat	1-Lying on Side	2-Lying Prone	3-Sitting or standing	4-Rearing on hind legs	5-Repeated vertical leaping
	Spontaneous Activity	0-None, resting	1-Casual groom, slow movement	2-Vigorous groom, moderate movement	3-Vigorous, rapid/dart movement	4-Extremely vigorous, rapid/dart movement	
	Respiration Rate	0-Gasping, irregular	1-Slow, shallow	2-Normal	3-Hyperventilation		
	Tremor	0-None	1-Mild	2-Marked			
	Urination	Count					
	Defecation	Count					
Arena	Transfer Arousal	1-Prolonged freeze, slight movement	2-Extended freeze, moderate movement	3-Brief freeze, active movement	4-Barely freeze, immediate movement	5-No freeze, immediate movement	6-Extremely excited (manic)
	Locomotor Activity	Squares entered within 30s					
	Palpebral Closure	0-Eyes wide open	1-Eyes 1/2 closed	2-Eyes closed			
	Piloerection	0-None	1-Coat stood on end				
	Startle Response	0-None	1-Preyer reflex-flick of pinnae	2-Jump <1cm	3-Jump >1cm		
	Gait	0-Normal	1-Fluid but abnormal	2-Limited movement only	3-Incapacity		
	Pelvic Elevation	0-Markedly flattened	1-Barely touches	2-Normal (3mm)	3-Elevated (>3mm)		
	Tail Elevation	0-Dragging	1-Horizontally Extended	2-Elevated			
	Touch Escape	0-No response	1-Mild (escape to firm stroke)	2-Moderate (rapid response to light stroke)	3-Escape response to approach		
Held by Tail	Positional Passivity	0-Struggles when held by tail	1-Struggles when held by scruff	2-Struggles when laid supine	3-Struggles when held by hind legs	4-No struggle	
	Trunk Curl	0-present	1-absent				
	Limb grasping	0-present	1-absent				
	Abnormal Behavior	0-present	1-absent				
	Visual Placing	0-None	1-Upon nose contact	2-Upon vibrassa contact	3-Before contact-18mm	4-Early extension-25mm	
	Whisker Brush	0-No response	1-Response to vigorous brush	2-Response to mild brushing	3-Response to initial touch		
	Whisker placement	0-None	1-Upon head contact	2-Upon vibrassa contact	3-Before vibrassa contact		
Reflex	Grip Strength	0-None	1-Slight grip, semi-effective	2-Moderate grip, effective	3-Active grip, effective	4-Unusually effective	
	Body Tone	0-Flaccid, no return to normal	1-Slight resistance	2-Extreme resistence, boardlike			
	Pinna reflex	0-None	1-Active reaction, brisk flick	2-Hyperactive, repetitive flick			
	Corneal reflex	0-None	1-Active single eye blink	2-Multiple eye blink			
	Toe Pinch	0-None	1-Slight withdrawal	2-Moderate withdrawal, not brisk	3-Brisk, rapid withdrawal	4-Rapid extension/flexion	
	Wire Manoeuvre	0-Active grip with hindlegs	1-Difficulty grasp with hindlegs	2-Unable to grasp with hindlegs	3-Unable to lift hindlegs, falls	4-Fall immediately	
Supine Restraint	Heart Rate	0-Slow, bradycardia	1-Normal	2-Fast, tachycardia			
	Limb Tone	0-No resistence	1-Slight resistence	2-Moderate resistence	3-Marked resistence	4-Extreme resistence	
	Abdominal Tone	0-Flaccid, no return to normal	1-Slight resistence	2-Extreme resistence, boardlike			
	Salivation	0-None	1-Slight margin of submax	2-Wet zone entire sub-max			
	Provoked Biting	0-Absent	1-Present				
	Penlight Vision	0-No response	1-Pupil dilation	2-Rapid blinking			
Other	Righting Reflex	0-No impairment	1-Lands on side	2-Lands on back	3-Fails to right when on back		
	Contact Righting Reflex	0-Absent	1-Present				
	Negative Geotaxis	0-Turns and climbs on grid	1-Turns but then freezes	2-Moves, but fails to turn	3-Does not move w/in 30s	4-Falls off	
	Fear	0-None	1-Freeze with transfer arousal				
	Irritability	0-None	1-Struggle with supine restraint				
	Aggression	0-None	1-Provoked biting or attack				
	Vocalization	0-None	1-Provoked during handling				

Table S2. List of tests performed during the SHIRPA primary screen and the scoring criteria for each test.

A

A	5′–TCGAATCCGCATTGACAGCCTCT–3′
B	5′–AAAGTTCAGGCCCTTCTGGT–3′
C	5′–AGTACAACCTGCGCTCACGC–3′
D	5′–TGATGCTGCAGTTCTGGGAGC–3′
E	5′–ACAACCTAGTCACCCGCTCCTA–3′
F	5′–ATGTGTCTGCCCCTGAAAAC–3′
NeoF	5′–TGCTCCTGCCGAGAAAGTAT–3′
NeoR	5′–AAGCGAAGGAGCAAAGCTGCTA–3′

B

Antigen	Antibody	Species	Company	Western Blot	IHC
Actin	Polyclonal	Goat	Santa Cruz (sc-1616)	1:1000	
Caspase-3	Monoclonal	Rabbit	Cell Signaling (9664)		1:100
Ctip2	Monoclonal	Rat	Abcam (ab18465)		1:500
Cux1	Polyclonal	Rabbit	Santa Cruz (sc-13024)		1:100
GFAP	Monoclonal	Mouse	BD Pharmingen (556327)		1:800
Lamin A	Monoclonal	Mouse	Millipore (MAB3540)		1:400
Lamin A/C	Polyclonal	Goat	Santa Cruz (sc-6215)	1:400	
Lamin B1	Polyclonal	Goat	Santa Cruz (sc-6217)	1:400	1:400
Lamin B2	Monoclonal	Mouse	Invitrogen (33-2100)	1:400	
Lamin C	Polyclonal	Rabbit	Lifespan Biosciences (LS-B2972)		1:200
NeuN	Monoclonal	Mouse	Millipore (MAB377)		1:500
Olig2	Polyclonal	Rabbit	Millipore (AB9610)		1:500
LAP2β	Monoclonal	Mouse	BD Biosciences (611000)		1:400
Emerin	Monoclonal	Mouse	Novocastra (NCL-EMERIN)		1:200
SUN1	Polyclonal	Rabbit	Abcam (ab74758)		1:800
Nup98	Monoclonal	Rat	Abcam (ab50610)		1:250

Table S3. List of primers and primary antibodies. (A) Sequences of the primers used in 5′ and 3′ long-range PCR reactions and genotyping PCR reactions for the *Lmna*$^{\text{PLAO-5NT}}$ and *Lmna*$^{\text{PLAO-UTR}}$ alleles. (B) List of primary antibodies used in this study.