Mutations in the Matrin 3 gene cause familial amyotrophic lateral sclerosis

Janel O Johnson[1,28], Erik P Pioro[2,28], Ashley Boehringer[3,28], Ruth Chia[4,28], Howard Feit[5], Alan E Renton[1], Hannah A Pliner[1], Yevgeniya Abramzon[1], Giuseppe Marangi[1,6], Brett J Winborn[7], J Raphael Gibbs[8,9], Michael A Nalls[10], Sarah Morgan[9], Maryam Shoai[9], John Hardy[9], Alan Pittman[9], Richard W Orrell[11], Andrea Malaspina[12], Katie C Sidle[9], Pietro Fratta[13], Matthew B Harms[14], Robert H Baloh[15], Alan Pestronk[14], Conrad C Weihl[14], Ekaterina Rogaeva[16], Lorne Zinman[17], Vivian E Drory[18], Giuseppe Borghero[19], Gabriele Mora[20], Andrea Calvo[21], Jeffrey D Rothstein[22], ITALSGEN[23], Carsten Drepper[24,25], Michael Sendtner[24], Andrew B Singleton[10], J Paul Taylor[7], Mark R Cookson[4], Gabriella Restagno[26,29], Mario Sabatelli[27,29], Robert Bowser[3,29], Adriano Chiò[21,29] & Bryan J Traynor[1,22,29]

MATR3 is an RNA- and DNA-binding protein that interacts with TDP-43, a disease protein linked to amyotrophic lateral sclerosis (ALS) and frontotemporal dementia. Using exome sequencing, we identified mutations in *MATR3* in ALS kindreds. We also observed MATR3 pathology in ALS-affected spinal cords with and without *MATR3* mutations. Our data provide more evidence supporting the role of aberrant RNA processing in motor neuron degeneration.

ALS is a devastating neurodegenerative disease characterized by progressive paralysis and respiratory failure leading to death. Much attention has focused on the discovery of causal genes on the basis that understanding the pathophysiology underlying motor neuron degeneration would provide rational targets for therapeutic development. The genetic etiology of two-thirds of the familial form of ALS and 11% of the more common sporadic form of the disease are now known[1]. Nevertheless, the discovery of more genes would allow more complete mapping of the cellular pathways underlying this condition.

Here we applied exome sequencing to a family of European ancestry in which several individuals had been diagnosed with ALS and dementia (**Fig. 1a**) with the aim of identifying the causative mutation. We found two previously unknown, heterozygous missense variants that segregated with disease in this kindred, namely Ala436Val (chr5:126156748, C>T) in *LMNB1* and Phe115Cys (chr5:138643448, *T* >G) in *MATR3*. Neither variant was present in population polymorphism databases (including the Exome Sequencing Project ($n = 13,000$ control chromosomes), the 1000 Genomes Project ($n = 2,184$ chromosomes) and dbSNP) or in the Human Gene Diversity Panel ($n = 2,102$ chromosomes screened in our laboratory). The *MATR3* variant was also not present in another 5,190 neurologically normal subjects genotyped in our laboratory, bringing the total number of control chromosomes that did not carry this transversion to 27,666.

A Ser85Cys (chr5:138643358, C>G) mutation in *MATR3* was previously reported as the cause of autosomal dominant, distal, asymmetrical myopathy with vocal cord paralysis in a large, multigenerational family (**Fig. 1b**)[2,3]. Neurophysiological studies and muscle biopsies of affected members were variably reported to be consistent with either a neurogenic or a myopathic pattern.

In light of our genetic findings, the senior author (B.J.T.) and the neurologist who initially reported this family (H.F.) reevaluated the Ser85Cys *MATR3* family. Affected individuals developed progressive respiratory failure resulting in death, typically after 15 years of illness. Pathologically brisk knee reflexes, indicative of upper motor neuron lesions, were present in four of six patients examined. One patient also had brisk upper limb reflexes, as well as tongue fasciculations and a brisk jaw jerk. All of the examined cases displayed a 'split-hand' pattern of weakness suggestive of a lesion in the anterior horn of the cervical spinal cord, a sign commonly observed in ALS patients[4].

[1]Neuromuscular Diseases Research Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA. [2]Department of Neurology, Neuromuscular Center, Neurological Institute, Cleveland Clinic, Cleveland, Ohio, USA. [3]Division of Neurology, Barrow Neurological Institute, Phoenix, Arizona, USA. [4]Cell Biology and Gene Expression Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA. [5]Department of Neurology, Henry Ford Hospital, Detroit, Michigan, USA. [6]Institute of Medical Genetics, Catholic University of Sacred Heart, Rome, Italy. [7]Department of Cell and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. [8]Computational Biology Core, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA. [9]Department of Molecular Neuroscience and Reta Lila Weston Laboratories, Institute of Neurology, University College London, London, UK. [10]Molecular Genetics Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA. [11]Department of Clinical Neuroscience, Institute of Neurology, University College London, London, UK. [12]Centre for Neuroscience and Trauma, Blizard Institute, Queen Mary University of London, North-East London and Essex Regional Motor Neuron Disease Care Centre, London, UK. [13]Department of Neurodegenerative Disease, University College London, London, UK. [14]Department of Neurology, Washington University School of Medicine, St. Louis, Missouri, USA. [15]Department of Neurology, Cedars-Sinai Medical Center, Los Angeles, California, USA. [16]Tanz Centre for Research of Neurodegenerative Diseases, Division of Neurology, Department of Medicine, University of Toronto, Toronto, Ontario, Canada. [17]Division of Neurology, Department of Internal Medicine, Sunnybrook Health Sciences Centre, University of Toronto, Toronto, Ontario, Canada. [18]Department of Neurology, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel. [19]Department of Neurology, Azienda Universitaria-Ospedaliera di Cagliari and University of Cagliari, Cagliari, Italy. [20]ALS Center, Salvatore Maugeri Foundation, Milan, Italy. [21]'Rita Levi Montalcini' Department of Neuroscience, University of Turin, Turin, Italy. [22]Brain Science Institute, Department of Neurology, Johns Hopkins University, Baltimore, Maryland, USA. [23]Full lists of members and affiliations appear in the **Supplementary Note**. [24]Institute for Clinical Neurobiology, University of Würzburg, Würzburg, Germany. [25]Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Würzburg, Germany. [26]Molecular Genetics Unit, Department of Clinical Pathology, Azienda Sanitaria Ospedaliera Ospedale Infantile Regina Margherita-Santa Anna, Turin, Italy. [27]Neurological Institute, Catholic University and Insieme Contro le Malattie del Motoneurone Association for ALS Research, Rome, Italy. [28]These authors contributed equally to this work. [29]These authors jointly directed this work. Correspondence should be addressed to B.J.T. (traynorb@mail.nih.gov).
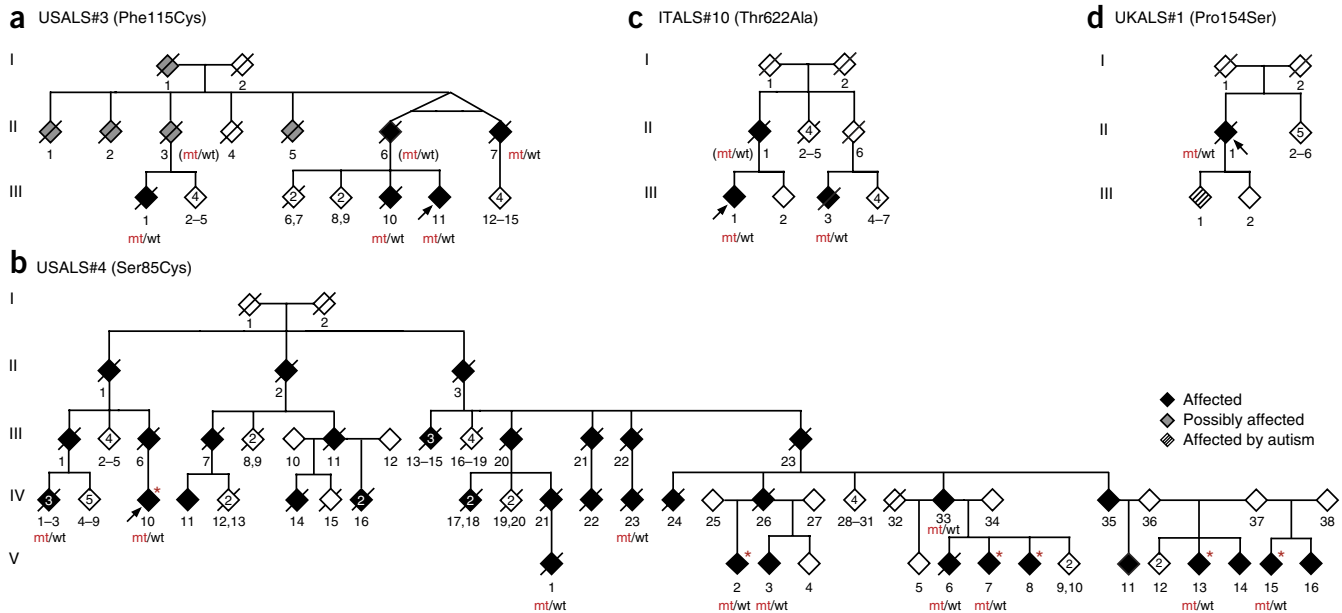
Figure 1 Pedigrees of patients with *MATR3* mutations. (**a**) Kindred USALS#3. (**b**) Kindred. (**c**) Kindred ITALS#10. (**d**) Kindred UKALS#1. mt, mutant alleles; wt, wild-type alleles[3]. Genotypes of presumed obligate carriers are in brackets. Red asterisks indicate individuals who underwent clinical examination. Arrows denote probands.

These clinical findings supported reclassification of this condition as slowly progressive ALS, and the presence of upper motor neuron signs in the form of brisk reflexes ruled out myopathy as the only cause of disease in this family.

To determine the frequency of *MATR3* mutations as a cause of ALS, we examined exome sequence data from 108 additional familial ALS cases. We identified a Thr622Ala (chr5:138658372, A>G) missense change in *MATR3* in a 66-year-old Sardinian diagnosed with familial ALS. This variant was present in a first cousin, who had also presented with typical, rapidly progressive ALS at the age of 64 (**Fig. 1c**). In addition, custom resequencing of genes linked to neurodegeneration in 96 British ALS cases identified a Pro154Ser (chr5:138643564, C>T) missense variant in *MATR3* in an individual diagnosed with sporadic disease (**Fig. 1d** and **Supplementary Fig. 1**). Again, neither mutation was present in population polymorphism databases or in the Human Gene Diversity Panel ($n = 17,286$ control chromosomes). More studies are required to confirm the pathogenicity of these variants, especially Pro154Ser, which was found in a single sporadic case and consequently lacks segregation data. We did not find any more mutations in the *LMNB1* gene.

We examined subcellular distribution of MATR3 using immunohistochemistry. In control subjects, we detected MATR3 in a granular staining pattern in the nuclei of motor neurons and surrounding glial cells (**Supplementary Fig. 2a**). In ALS patients, we observed MATR3 in the nuclei of remaining motor neurons and occasionally in the cytoplasm (**Supplementary Fig. 2b**). In a patient harboring the Phe115Cys *MATR3* mutation, MATR3 immunoreactivity was intense in the nucleus of all motor neurons and diffuse cytoplasmic staining was evident

in many neurons (**Supplementary Fig. 2c**). Cytoplasmic inclusions were absent in this individual. However, we detected rare MATR3-positive cytoplasmic inclusions in an ALS patient known to carry the *C9ORF72* repeat expansion (**Supplementary Fig. 3**).

MATR3 is a 125-kDa nuclear matrix protein that binds DNA and RNA. Previous unbiased screens found that MATR3 protein interacts with TDP-43, an RNA-binding protein whose mutation is known to cause ALS[5,6]. To confirm this interaction, we performed coimmunoprecipitation of Flag-tagged MATR3 variants with endogenous TDP-43 in human embryonal kidney cells transformed with the SV40 large T antigen (HEK293FT cells). As the genetic data were strongest for the Phe115Cys and Ser85Cys mutations, we selected these variants for further scrutiny, as well as the Thr622Ala
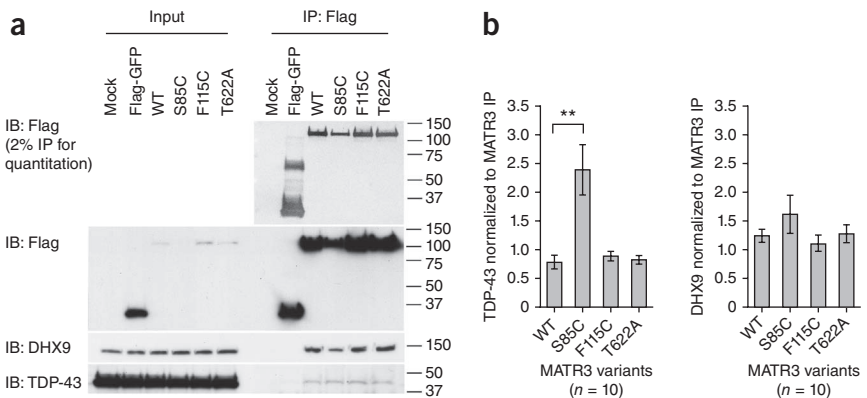

Figure 2 Immunoprecipitation of MATR3 with TDP-43. (**a**) Flag-MATR3 was expressed in HEK293FT cells, immunoprecipitated using anti-Flag antibody and probed with TDP-43 and DHX9 antibodies. Untransfected cells, Mock; cells transfected with wild-type Flag-MATR3 construct, WT; cells transfected with Flag-MATR3 construct carrying the Ser85Cys mutation, S85C; carrying the Phe115Cys mutation, F115C; carrying the Thr622Ala mutation, T622A. Molecular weight (in kDa) of protein marker is shown to the right of the blots. (**b**) Graphs show ratio of immunoprecipitated TDP-43 or DHX9 to Flag-MATR3; mean ± s.e.m. based on 10 replicate immunoprecipitation experiments. Differences in interaction between MATR3 and TDP-43 were tested with Wilcoxon signed rank test; **$P < 0.01$. Full-length blots are presented in **Supplementary Figure 10**.

variant, for which proof of pathogenicity was less clear. We found a reliable interaction that, interestingly, was increased by the Ser85Cys mutation but not by the Phe115Cys or Thr622Ala variants (**Fig. 2**). We also noted that Ser85Cys MATR3 was expressed at lower steady-state levels than other variants, suggesting a structural effect of the mutation (**Fig. 2**). There was no alteration in interaction between MATR3 and a second protein interactor, DHX9, demonstrating that the effect of Ser85Cys is specific for TDP-43 and not generalized to all interactions of MATR3. Both interactions were abolished by RNase (**Supplementary Fig. 4**), demonstrating that they are RNA dependent. Coimmunoprecipitation of endogenous protein confirmed that MATR3 and TDP-43 interact at endogenous abundances (**Supplementary Fig. 5**). Also in accord with an interaction, MATR3 and TDP-43 coaggregated in skeletal muscle tissue of a patient carrying the Ser85Cys mutation (**Supplementary Fig. 6**).

Differential effects of different mutations in the same gene have been reported for other neurodegenerative diseases, such as familial Parkinson's disease caused by *LRRK2* mutations[7] and familial ALS caused by *FUS* mutations[8]. Therefore, the lack of an effect of the Phe115Cys and Thr622Ala mutations on the MATR3–TDP-43 interaction does not necessarily preclude their pathogenicity, as it is possible that these mutations disrupt other cellular processes in a manner that would not be detected by our assays. The variants found in *MATR3* were distributed along the length of protein, perhaps disrupting different nearby domains. Indeed, several functions have been associated with *MATR3*, including RNA processing[6], retention of hyper-edited RNA[9], gene silencing through interaction with Argonaute-containing complexes[10], chromatin organization[11] and mediation of neuronal cell death in response to NMDA-type glutamate receptor activation[12]. The Ser85Cys variant only altered interaction with TDP-43 and not another MATR3 partner, DHX9. However, and reminiscent of mutations in VAPB[13], Ser85Cys MATR3 is notably less stable than other MATR3 variants. We infer that, of the genetic variants tested, Ser85Cys has the strongest effect on protein structure and that this is correlated with a change in affinity for TDP-43. The structural basis of this interaction will need to be resolved in future studies.

We note that the Ser85Cys mutation in MATR3 was associated with slowly progressive form of ALS, whereas individuals carrying the Phe115Cys-encoding mutation typically died from respiratory failure within 5 years of symptom onset. Similar phenotype variability has been observed for other ALS-associated genes. For example, the Ala4Val-encoding mutation of superoxide dismutase 1 is associated with an aggressive form of the disease with an average survival of only 9 months after symptom onset[14]. In contrast, the homozygous Asp90Ala-encoding mutation in the same gene is associated with an indolent course, with patients developing respiratory failure after 10 years of illness[14]. Furthermore, the phenotype observed in some patients carrying *MATR3* mutations combined features of ALS and myopathy. This clinical pattern is markedly similar to that observed in patients with mutations in *VCP*, *HNRNPA1* and *HNRNPA2B1*, and the term "multisystem proteinopathy" has been used to reflect this broad pleiotropy[15,16].

Exome sequencing data from the original family, as well as the 108 familial ALS cases, have been made available on dbGaP (accession code phs000101). The public release of such data allows other ALS researchers around the world to access, reanalyze and combine it with their own sequence data, thereby accelerating the pace of gene discovery.

In summary, our genetic data identify mutations of the *MATR3* gene as a rare cause of familial ALS and broaden the phenotype associated with this gene beyond the previously reported distal myopathy. This provides further insight into the importance of RNA metabolism in this fatal neurodegenerative disease.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. dbGaP: phs000101.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

1. Renton, A.E., Chiò, A. & Traynor, B.J. *Nat. Neurosci.* **17**, 17–23 (2014).
2. Feit, H. *et al. Am. J. Hum. Genet.* **63**, 1732–1742 (1998).
3. Senderek, J. *et al. Am. J. Hum. Genet.* **84**, 511–518 (2009).
4. Eisen, A. & Kuwabara, S. *J. Neurol. Neurosurg. Psychiatry* **83**, 399–403 (2012).
5. Ling, S.C. *et al. Proc. Natl. Acad. Sci. USA* **107**, 13318–13323 (2010).
6. Salton, M. *et al. PLoS ONE* **6**, e23882 (2011).
7. Cookson, M.R. *Biochem. Soc. Trans.* **40**, 1070–1073 (2012).
8. van Blitterswijk, M. *et al. PLoS ONE* **8**, e60788 (2013).
9. Zhang, Z. & Carmichael, G.G. *Cell* **106**, 465–475 (2001).
10. Höck, J. *et al. EMBO Rep.* **8**, 1052–1060 (2007).
11. Ma, H., Siegel, A.J. & Berezney, R. *J. Cell Biol.* **146**, 531–542 (1999).
12. Giordano, G. *et al. J. Neurochem.* **94**, 808–818 (2005).
13. Aliaga, L. *et al. Hum. Mol. Genet.* **22**, 4293–4305 (2013).
14. Chiò, A. *et al. Amyotroph. Lateral Scler.* **10**, 310–323 (2009).
15. Johnson, J.O. *et al. Neuron* **68**, 857–864 (2010).
16. Kim, H.J. *et al. Nature* **495**, 467–473 (2013).

ONLINE METHODS

Description of the USALS#3 pedigree (Phe115Cys). The proband (III:11, **Fig. 1a**) developed dysarthria at 50 years of age. He developed gait difficulties because of leg stiffness and cramping in the feet. He began choking on liquids at age 51 and developed pseudobulbar affect but no detectable cognitive impairment. Nocturnal noninvasive ventilation was prescribed for obstructive sleep apnea since age 53. Neurological examination at 52 years revealed mild, predominantly spastic dysarthria; increased jaw jerk; brisk facial reflexes, especially on the right; and slowed lateral tongue movements. Mild muscle atrophy was noted in the right thigh and rare fasciculations were seen in the proximal upper limbs. Tone was mildly spastic in the right arm and lower limbs, with slowed right fine finger movements and right foot tapping. Power in upper and lower limbs was normal. Tendon reflexes were pathologically brisk throughout, with right extensor plantar response. Gait and sensation were normal.

Dysarthria, dysphagia, and distal weakness in upper and lower extremities progressed slowly over 2 years, requiring use of bilateral ankle foot orthoses. Most recent neurologic examination at age 55 revealed mild cognitive impairment with disinhibition and inability to vocalize (anarthria). Vertical saccades were slowed and upgaze was noticeably limited. Jaw jerk was markedly brisk with clonus, as were facial reflexes, and there was a hyperactive gag reflex. The tongue was severely atrophic, fasciculating, and unable to move off the midline or protrude beyond the lower teeth. Muscle atrophy was global, being moderate proximally and severe distally, especially in intrinsic hand muscles and forelegs. Fasciculations were active in both upper limbs and neck regions, and tone was markedly spastic in the arms and legs, particularly over the right side. Weakness was most prominent distally in hands and feet, with little or no movement of thumb and fingers (0–2/5) and ankle and toes (0–2/5). Proximal upper and lower limb strength was better (4/5), allowing the patient to stand, although shoulder pain from contractions limited useful arm function. Tendon reflexes were pathologically brisk throughout, more marked on the right with ipsilateral extensor plantar response. He was unable to walk because of the weakness and spasticity, and used a wheelchair for mobility.

Patient II:6. The mother of the proband (II:6, **Fig. 1a**) developed bilateral finger and hand weakness at age 70. This was followed a few months later by speech and swallowing problems, and ALS was diagnosed at age 71. Her father had died at 47 years of age after a prolonged course involving leg muscle weakness. Three siblings died in their eighties: one (II:1) died of dementia, another (II:2) developed limb weakness and was unable to walk at the time of death, and a third (II:5) died of dementia and also was reported to be dysarthric and to have upper limb weakness.

Neurological examination at 71 years revealed normal speech and cognition, brisk facial reflexes, mentalis muscle fasciculations and weak (4/5) neck flexors. Fasciculations were seen in both upper extremities and thighs, with moderate atrophy of intrinsic hand muscles. She was weaker distally, with thumb abductors, thumb flexors and intrinsic hand muscles 3/5, shoulder abductors 4/5, ankle dorsiflexors and toe extensors 4–/5, and hip flexors 4/5. Tendon reflexes were pathologically brisk, with a right extensor plantar response. Gait was unsteady and partially steppage in nature. Over the next 3 years, her limbs became progressively paralyzed and she developed dysarthria and mild cognitive impairment. She died of respiratory failure at 75 years of age.

Patient II:7. An aunt of the proband (II:7, **Fig. 1a**) and identical twin of the proband's mother developed slowly progressive hand weakness at 57 years of age. Four years later, she began experiencing dysarthria, at which point she was diagnosed with ALS. Her weakness gradually spread to the rest of her limbs and she developed severe cognitive impairment. Currently, at age 82, she is unable to stand or walk and uses a wheelchair for mobility. Her hand weakness has deteriorated to the point that she cannot feed herself or brush her teeth, and she is aphasic. She has respiratory failure (forced vital capacity 49%).

Patient III:10. The proband's older sibling (III:10, **Fig. 1a**) developed right (dominant) hand stiffness and cramping at 52 years of age. At 57 years, he began experiencing progressive weakness of right hand and finger muscles resulting in inability to button or tie shoelaces. Atrophy of intrinsic right hand muscles was also noted. The patient denied leg weakness but would develop cramping of thigh muscles after squatting. About 18 months later the patient

noticed worsening left hand weakness. There were no bulbar symptoms or shortness of breath initially.

Neurological examination at age 58 revealed no cognitive or behavioral abnormalities, normal speech, and no bulbar signs except for slightly increased right facial reflex and right palmomental reflex. Upper limbs showed moderate atrophy of distal muscles, especially of the lateral hand in a split hand pattern, with continuous fasciculation of right more than left shoulder and arm muscles. Lower limbs showed no changes. Tone was normal throughout. Weakness was most prominent distally and slightly worse on the right, with thumb abductors and deep finger flexors 3/5, finger abductors and extensors 4/5, wrist flexors and extensors 4+/5, and elbow flexors and extensors almost 5/5. Tendon reflexes were pathologically brisk throughout, with left extensor plantar response. Over the next 2 years, the patient's condition gradually deteriorated, with progressive limb and respiratory muscle weakness. The patient expired 2 years later from respiratory failure at 60 years of age. Brain and spinal cord were obtained for pathologic analysis.

Patient III:1. A cousin of the proband (III:1, **Fig. 1a**) initially presented with lower limb weakness at 63 years of age. Symptoms progressed to involve the remaining limbs and bulbar musculature. Severe dysphagia necessitated the placement of a gastrostomy tube. The patient's mother (II:3) died of dementia in her eighties. The patient became cognitively impaired several years after the initial presentation and died from respiratory failure at the age of 68.

Affected members of the USALS#3 kindred were negative for the pathogenic repeat expansion of *C9ORF72.*

Description of the USALS#4 pedigree (Ser85Cys). The proband of the USALS#4 family (IV:10, **Fig. 1b**) developed right foot drop at 44 years of age. Muscle weakness spread to the remaining limbs over the next 2 years. A diagnosis of Charcot-Marie-Tooth disease was made at the age 46 on the basis of a neurogenic pattern observed in an electromyogram and nerve conduction studies (EMG/NCS). Reevaluation 2 years later led to the patient's illness being reclassified as non-Scandinavian distal myopathy. The patient's condition progressed, with the development of dysarthria and mild dysphagia by the age of 53 and respiratory failure requiring nocturnal noninvasive ventilation by the age of 56. Currently, at age 65, the subject uses a power wheelchair for mobility.

Neurological examination at age 65 revealed mild dysarthria, perioral fasciculations, a brisk jaw jerk and moderately weak neck extension. There was generalized muscle wasting and pyramidal-distribution weakness in all four limbs. In the upper limbs, shoulder abduction was 3/5 bilaterally, elbow extension was 4/5, wrist extension and finger extension were 1/5, and finger abduction, finger adduction and thumb abduction were 3/5, whereas shoulder adduction, elbow flexion, wrist flexion and abductor digiti minimi were 5/5 bilaterally. In the lower limbs, hip flexion was 4/5 bilaterally; ankle dorsiflexion, ankle plantar flexion and extensor hallucis longus were 0/5, whereas hip abduction, hip adduction, knee flexion and knee extension were 5/5 bilaterally. Tendon reflexes were absent, and toes were mute on Babinski testing. Proprioception and vibration sensation were diminished to the level of the ankles and knees, respectively, bilaterally. The patient was able to stand and walk slowly using a walker and with the assistance of one person.

Patient V:2. A cousin of the proband (V:2, **Fig. 1b**) noticed right foot drop at 42 years of age. Within 5 years, the muscle weakness had spread to involve both hands. The patient developed dysarthria and mild dysphagia at age 47 and respiratory failure requiring supplemental oxygen and noninvasive ventilation at 49. The patient had an episode of aspiration pneumonia at age 55 that required prolonged intubation and ventilation. Currently, at age 57, the patient uses a power wheelchair but can walk slowly using a walker.

Neurological examination at age 57 revealed mild dysarthria, mild facial weakness, poor palate elevation and a brisk jaw jerk. Limb examination showed generalized muscle atrophy and a pyramidal pattern of weakness. In the upper limbs, shoulder abduction and elbow extension were 4/5 bilaterally, wrist dorsiflexion was 1/5, finger extension and thumb abduction were 3/5, and finger flexion and abductor digiti minimi were 4/5, whereas shoulder adduction, elbow flexion and wrist flexion were 5/5 bilaterally. In the lower limbs, hip flexion was 4/5 bilaterally, and ankle dorsiflexion, ankle plantar flexion, toe flexion and toe extension were 1/5, whereas hip abduction, hip adduction, knee flexion and

knee extension were 5/5 bilaterally. Deep tendon reflexes were absent in the upper limbs. The right knee jerk was 2+, and the left knee jerk was brisk (3+). Ankle jerks were absent and toes were mute on Babinski testing. There was loss of pinprick and temperature sensation to the mid-calf level bilaterally.

Patient V:7. A cousin of the proband (V:7, **Fig. 1b**) observed right leg weakness at 33 years of age. Symptoms progressed to the point that the patient has been using a power wheelchair since 57, although continuing to walk slowly using a rollator walker as part of an exercise regimen. Hand weakness developed at age 60, and the subject had an episode of aspiration pneumonia requiring prolonged intubation and hospitalization at age 63. The patient has required nocturnal noninvasive ventilation and daytime oxygen supplementation since that time. Mild dysphagia and occasional choking episodes required changes in food consistency.

Neurological examination at age 65 revealed mild dysarthria, mild facial weakness and a brisk jaw jerk. Generalized limb atrophy and pyramidal-distribution weakness was evident. In the upper limbs, shoulder abduction, elbow flexion and elbow extension were 4/5 bilaterally, wrist extension was 3/5, finger extension was 4/5 and thumb abduction was 3/5, whereas shoulder adduction, wrist flexion and adductor digiti minimi were 5/5 bilaterally. In the lower limbs, hip flexion was 4/5 bilaterally, hip abduction and hip adduction were 3/5, knee flexion and knee extension were 1/5, ankle dorsiflexion and ankle plantar flexion were 0/5, and toe extension was 1/5 bilaterally. Deep tendon reflexes were absent in all four limbs, and toes were mute on Babinski testing. Temperature sensation was diminished to the mid-calf level bilaterally.

Patient V:8. A cousin of the proband (V:8, **Fig. 1b**) presented with dysarthria and choking at 47 years of age. The patient developed left ankle weakness at age 52. Weakness had spread to both hands by age 58. Currently, at age 63, the patient has difficulty using eating utensils and walks with the aid of a walker.

Neurological examination at age 63 revealed dysarthria with nasal air escape, poor palate elevation, tongue fasciculations and a brisk jaw jerk. There was marked distal atrophy and weakness. In the upper limbs, wrist extension was 4/5 bilaterally, finger extension and thumb abduction were 3/5, and left abductor digiti minimi was 4/5, whereas bilateral shoulder abduction, shoulder adduction, elbow flexion, elbow extension, wrist flexion and right adductor digiti minimi were 5/5. In the lower limbs, ankle dorsiflexion and ankle invertors were 3/5 bilaterally and extensor hallucis longus was 4/5, whereas hip flexion, hip extension, hip abduction, hip adduction, knee flexion, knee extension, ankle plantar flexion and foot evertors were 5/5 bilaterally. Triceps reflexes were brisk (3+) bilaterally, whereas other deep tendon reflexes in the upper limbs were normal (2+). Knee jerks were brisk (3+) bilaterally, ankle jerks were absent, and toes were mute on Babinski testing. Vibration sensation was diminished to the level of the ankles bilaterally.

Patient V:13. A cousin of the proband (V:13, **Fig. 1b**) noticed mild dysarthria and throat-clearing difficulties at 42 years of age. At age 44, the subject developed right leg weakness that spread to involve the left leg and both hands by age 51. The patient occasionally chokes when eating. Currently, at age 58, the patient remains mobile with the aid of bilateral ankle orthotics.

Neurological examination, at age 58, revealed trace dysarthria. There was prominent distal muscle atrophy. In the upper limbs, right shoulder abduction was 4/5 bilaterally and wrist extension and finger extension were 3/5, whereas shoulder adduction, elbow flexion, elbow extension, wrist flexion and finger flexion were 5/5 bilaterally. In the lower limbs, left hip flexion was 4/5, ankle dorsiflexion was 2/5 bilaterally and extensor hallucis longus was 3/5, whereas hip extension, hip abduction, hip adduction, knee flexion, knee extension and ankle plantar flexion were 5/5 bilaterally. In the upper limbs, reflexes were diminished (1+). In the lower limbs, knee jerks were brisk (3+ with crossed adductors), ankle jerks were absent, and the toes were mute on Babinski testing. All sensory modalities were intact.

Patient V:15. A cousin of the proband (V:15, **Fig. 1b**) developed hand weakness at 49 years of age. Currently, at age 50, the patient complains of fatigue, frequent cramping of the right foot, and mild dysarthria when fatigued.

Neurological examination, at age 50, revealed bilateral thenar and first dorsal interossei muscle atrophy. In the upper limbs, wrist extension, finger extension and thumb abduction were 4/5 bilaterally, whereas shoulder abduction, shoulder

adduction, elbow flexion, elbow extension, wrist flexion and finger flexion were 5/5 bilaterally. In the lower limbs, extensor hallucis longus was 4/5 bilaterally, whereas all other muscle groups were 5/5. Reflexes in the upper limbs were 2+ with the exception of the left brachioradialis, which was diminished (1+). In the lower limbs, knee jerks were brisk (3+ with crossed adductors) bilaterally, ankle jerks were absent, and toes were downgoing on Babinski testing. Vibration sensation was diminished to the level of the ankle bilaterally. The patient had difficulty with heel walking and toe walking.

In summary, the clinical features of patients in the Ser85Cys *MATR3* kindred were consistent with a progressive, fatal motor neuron disease with combined upper and lower motor neuron signs, bulbar dysfunction and respiratory failure.

Description of the ITALS#10 pedigree (Thr622Ala). The proband (III:1, **Fig. 1c**) developed spinal ALS presenting with left foot drop at 62 years of age. MRI with diffusion tensor imaging revealed bilateral corticospinal tract damage. The patient was cognitively normal on exam, and [^{18}F]fluorodeoxyglucose PET imaging was also reported to be normal. The patient remains alive 32 months after symptom onset and uses noninvasive ventilation for 12 h per day.

Patient III:3. A cousin of the proband (III:3, **Fig. 1c**) presented with a 5-month history of progressive right arm weakness at 60 years of age. Neurological examination at the time of presentation showed weakness and hypotrophy in upper limbs, more evident on the right side and in distal muscles. Deep tendon reflexes were diffusely brisk, especially on the right. MRIs of the brain and cervical spinal cord were normal. EMG showed active and chronic denervation in upper limbs muscles, whereas motor evoked potentials revealed increased central conduction time. Over the following months, the patient's symptoms progressed to involve the lower limbs and respiratory muscles. She died 33 months after disease onset of respiratory failure.

No mutation in known ALS-associated genes, including the pathogenic repeat expansion of *C9ORF72*, segregated with disease in the ITALS#10 kindred.

Description of the UKALS#1 pedigree (Pro154Ser). The proband of the UKALS#1 family (II:1, **Fig. 1d**) was Indian and developed upper limb weakness at 59 years of age. There was no family history of ALS or dementia, though an offspring (III:I) was diagnosed with autism. Over the next 3 years, the patient's symptoms spread to involve the remaining limbs and bulbar musculature. The patient now uses a wheelchair for mobility and requires ventilator support for respiratory failure, a gastrostomy tube for feeding and an eye-tracking system for communication. Neurological examination, at age 62, revealed widespread upper and lower motor neuron signs and bulbar involvement consistent with a diagnosis of ALS.

The affected member of UKALS#1 was negative for mutations in known ALS-associated genes, including the pathogenic repeat expansion of *C9ORF72*.

Additional samples. For subsequent mutational screening, we examined exome sequence data that had been generated in our laboratory using DNA obtained from 108 individuals ($n = 6$ Canadians, $n = 14$ Germans, $n = 9$ Israelis, $n = 32$ Italians, $n = 47$ from the United States) who had been diagnosed with familial ALS and who were negative for mutations in known ALS-associated genes, including the pathogenic hexanucleotide repeat expansion of *C9ORF72*. Average age of symptom onset among this cohort was 55.1 (range, 15.0–79.0), and 47.2% were male.

Multiethnic control samples consisted of a series of 1,051 anonymous samples that are part of the Human Gene Diversity Panel (http://www.cephb.fr/en/hgdp/diversity.php/). These samples come from the following geographical regions: Africa ($n = 122$), Algeria ($n = 30$), Brazil ($n = 45$), Cambodia ($n = 11$), China ($n = 182$), Colombia ($n = 13$), France ($n = 53$), Israel ($n = 144$), Italy ($n = 50$), Japan ($n = 30$), Mexico ($n = 50$), New Guinea ($n = 39$), Orkney Islands ($n = 16$), Pakistan ($n = 199$) and Russia ($n = 67$).

Neurologically normal control subjects who were genotyped as part of the genome-wide association study that was ongoing in the Laboratory of Neurogenetics, NIA, consisted of samples from the United States ($n = 2,882$), the United Kingdom ($n = 677$), Italy ($n = 1,242$) and Finland ($n = 389$).

The appropriate institutional review board (National Institute on Aging Institutional Review Board protocol number 2003-081) approved the study, and informed consent was obtained from all subjects included in this study.

Exome sequencing and bioinformatic analysis pipeline. DNA from affected individuals II:7, III:1, III:10 and III:11 of the USALS#3 family was enriched using TruSeq technology (version 1.0) and paired-end sequenced on a Hiseq2000 sequencer according to the manufacturer's protocol (Illumina, San Diego, CA). This generated 8.0 gigabases (Gb) of alignable sequence data for individual II:7, 15.4 Gb for III:1, 7.8 Gb for III:10 and 9.7 Gb for III:11 (mean 10× coverage = 95.4%, range 94.3 to 98.0%; mean 30× coverage = 87.9%, range 85.1 to 93.1%). Exome sequence data from the additional 108 familial ALS samples were generated in a similar manner. Sample randomization was not performed and the researchers were not blinded to genotype. Statistical methods were not used to predetermine sample sizes, but rather our sample sizes were dictated by patient availability and are similar to those generally employed in the field.

Sequence alignment and variant calling were performed against the reference human genome (UCSC hg 19) using the Genome Analysis Toolkit (http://www.broadinstitute.org/gatk/). PCR duplicates were removed before variant calling using Picard software (http://picard.sourceforge.net/index.shtml).

A series of standard filters was applied to the exome sequence data generated for the USALS#3 family to identify the causative mutation. First variants and indels that were not shared by all four affected members were excluded. Next variants and indels that were homozygous or nonautosomal were excluded. Then synonymous and noncoding changes were identified using the SeattleSeq online tool (annotation 137, http://snp.gs.washington.edu/SeattleSeqAnnotation137/index.jsp) and filtered from the variant list. Under the hypothesis that the mutation underlying this rare familial disease was not present in the general population, SNPs identified in the Exome Sequencing Project (n = 6,500, http://evs.gs.washington.edu/, accessed 25 March 2013), the 1000 Genomes project (n = 1,092, 20110521 release, version 3, http://www.1000genomes.org/, accessed 25 March 2013) or dbSNP (build 137, http://www.ncbi.nlm.nih.gov/SNP/, accessed 25 March 2013) were filtered. As an additional step, variants and indels detected in the USALS#3 family were filtered against exome data generated in our laboratory for 200 neurologically normal control subjects. **Supplementary Figure 7** shows the number of variants filtered by each of these steps in the USALS#3 pedigree, and **Supplementary Figure 8** shows the genomic location of the two novel coding variants identified by exome sequencing.

Sanger sequencing using customized primers was performed to confirm the presence of the two remaining variants.

Exome data for the 108 familial ALS cases was processed in an identical manner.

Genotyping. Genotyping of the *LMNB1* (chr5:126156748, C>T, Ala436Val, NM_005573.3) and *MATR3* (chr5:138643448, T > G, Phe115Cys, NM_199189.2) variants was performed in the Human Gene Diversity Panel samples using Taqman SNP genotyping assays (Life Technologies Corp., Grand Island, NY, USA) on the 7900HT Fast Real Time PCR System according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA).

The *MATR3* chr5:138643448, T > G (Phe115Cys) and chr5:138643358, C>G (Ser85Cys) variants were assayed in an additional 5,190 neurologically normal control subjects of European ancestry as part of a genome-wide association project that was ongoing in the Laboratory of Neurogenetics, NIA, using the HumanExome+ SNP chip with custom content (Illumina).

Immunohistochemistry of spinal cord. Immunohistochemistry was performed on lumbar spinal cord from ALS patients (n = 16), neurologically normal controls (n = 6) and one ALS patient with the Phe115Cys *MATR3* mutation as follows: tissues were deparaffinized, rehydrated and subjected to antigen retrieval for 20 min in a steamer in pH 9 buffer (Dako Inc., Carpinteria, CA, USA) for the Sigma antibody, and in pH 6 citrate buffer (BioGenex Inc., Freemont, CA, USA) for the Abcam antibody. This was followed by 20 min incubation in buffer warmed to 100 °C. Next, slides were blocked in Superblock (ScyTek Laboratories Inc., West Logan, UT, USA) with avidin protein (Vector Laboratories Inc., Burlingame, CA, USA) for 1 h, after which primary antibody diluted in Superblock (Pierce Biotechnology, Rockford, IL, USA) with biotin (Vector Laboratories) was added for a second hour, with PBS washes in between. Slides were then incubated in biotinylated horseradish peroxidase (HRP)-conjugated secondary antibody (catalog number B-2004, 1:200 dilution, Vector Laboratories). The primary anti-MATR3 antibodies were HPA036565 (1:500 dilution, Sigma-Aldrich Corp., St. Louis, MO, USA) and ab151714 (1:500 dilution, Abcam PLC, Cambridge, MA, USA) (see **Supplementary Fig. 9** for characterization of these antibodies). Slides were incubated with Vectastain Elite ABC reagent (Vector Laboratories) for 30 min, washed in PBS, and developed for 5 min using Vector NovaRED Substrate Kit (Vector Laboratories). Finally, slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich) for 1 min, dehydrated, and mounted in Permount medium.

All pictures were taken with an Olympus BX40 light microscope, and images were acquired using a Nikon DS L2 digital camera. Image analysis was performed with Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA). All of the samples shown in **Figure 2** and **Supplementary Figures 2** and **3** were processed at the same time.

Immunohistochemistry of skeletal muscle. Cryostat sections of rapidly frozen skeletal muscle were processed in a standard fashion consistent with ref. 17. Immunocytochemistry for each antibody was performed on tissue from patients and compared with normal tissue controls processed simultaneously (**Supplementary Fig. 6**). Primary antibodies used in this study were TDP-43 rabbit polyclonal antibody (catalog number 10782-2-AP, ProteinTech Antibody Group, Chicago, Illinois, USA) and MATR3 mouse monoclonal (catalog number sc-81318, Santa Cruz, Santa Cruz, CA). Dilutions were both 1:1,000. Double-labeling immunofluorescence was performed as previously described using secondary antibodies conjugated to Alexa Fluor 488 and 594 (Invitrogen, catalog numbers A-21200 and A-21442, 1:200 dilution). Sections were examined using a fluorescence microscope (80i upright; Nikon) and charge-coupled device camera (EZ monochrome; Roper Industries) with deconvolution software analysis (NIS Elements; Nikon). Image processing and analysis were performed with NIS Elements 4.0 software and Photoshop CS3 (Adobe Systems Inc.).

Immunoprecipitation. Mutations (Ser85Cys, Phe115Cys and Thr622Ala) were introduced into Flag-tagged MATR3 cDNA plasmid (Addgene, Cambridge, MA, USA) using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA). All plasmids were sequence-verified. For Flag immunoprecipitation of MATR3, HEK293FT cells (Life Technologies Corp., Grand Island, NY, USA) transiently expressing Flag-MATR3 were lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM EDTA, 0.5% (v/v) NP-40, phosphatase (Thermo Scientific, number 78427) and protease inhibitor (Roche, catalog number 04693159001) for 30 min at 4 °C. Lysate was precleared with EZview Red Protein G Affinity Gel (Sigma-Aldrich) for 30 min at 4 °C, followed by immunopurification with EZview Red Anti-Flag M2 Affinity Gel (catalog number F2426, Sigma-Aldrich) for 2 h at 4 °C. Protein-gel complexes were washed four times with lysis buffer. MATR3 was eluted using Gentle Ag/Ab Elution Buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 30 min at room temperature.

Protein samples were prepared for SDS-PAGE in SDS sample buffer (Life Technologies) and boiled at 95 °C for 10 min before electrophoresis on 4–20% TGX gels (Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins were transferred to PVDF membranes using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with 5% non-fat milk OmniBlok (American Bioanalytical Inc., Natick, MA, USA). The following primary antibodies were used at the indicated dilutions: rabbit anti-MATR3 ab151714 (1:200, Abcam), rabbit anti-MATR3 HPA036564 (1:200, Sigma-Aldrich), mouse anti-Flag F1804 (1:5,000, Sigma-Aldrich), rabbit anti-TDP43 10782-2-AP (1:2,000, ProteinTech Group Inc., Chicago, IL, USA) and rabbit anti-DHX9 A300-855A (1:2,000, Bethyl Laboratories Inc., Montgomery, TX, USA). Immunoreactivity was revealed using appropriate HRP-conjugated secondary antibodies (1:5,000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, catalog numbers 711-035-1521 and 715-035-1501) and the ECL Plus chemiluminescent system (Pierce). Quantitation was performed using ImageJ software (version 1.41, National Institutes of Health, USA). Wilcoxon signed-rank test was used to estimate significance for differences in median values of interaction of MATR3 with TDP-43 and DHX9, comparing the values relative to wild-type protein in each experiment.

17. Weihl, C.C. *et al. J. Neurol. Neurosurg. Psychiatry* **79**, 1186–1189 (2008).