A novel variant of human SOD1 harboring ALS-associated and experimental mutations in metal-binding residues and free cysteines lacks toxicity in vivo.

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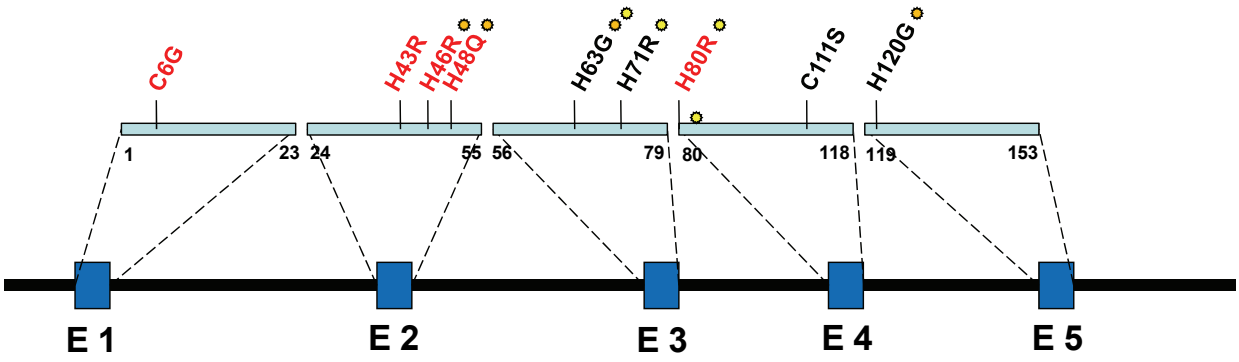
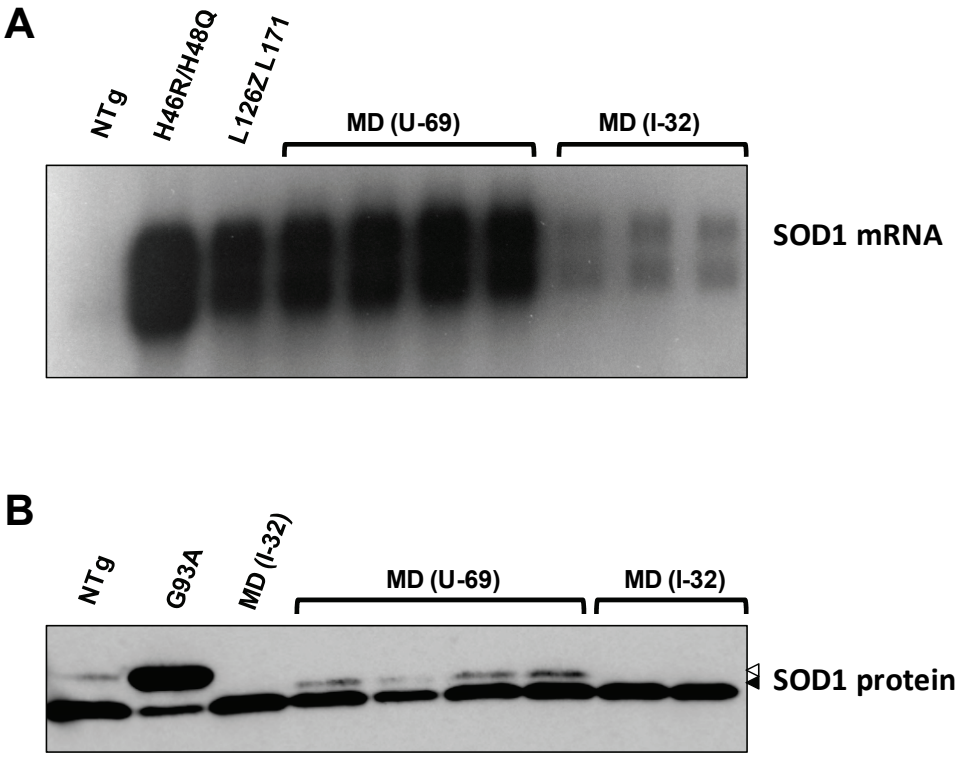
Supplemental Material

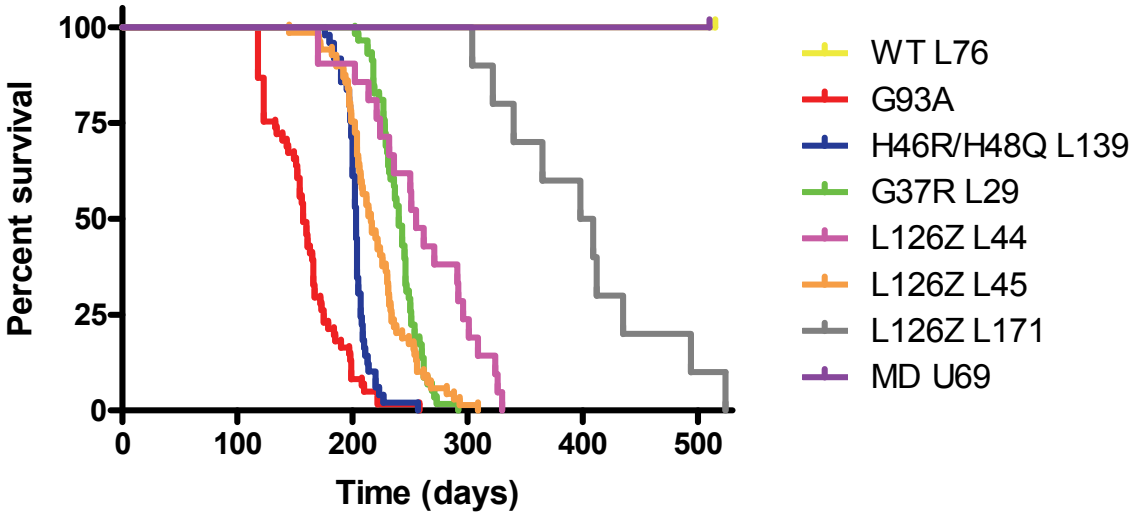


Exon #	First-Last amino acid	Mutation
Exon 1 (E 1):	1-23	C6G
Exon 2 (E 2):	24-55	H43R, H46R, H48Q
Exon 3 (E 3):	56-79	H63G, H71R
Exon 4 (E 4):	80-118	H80R, C111S
Exon 5 (E 5):	119-153	H120G

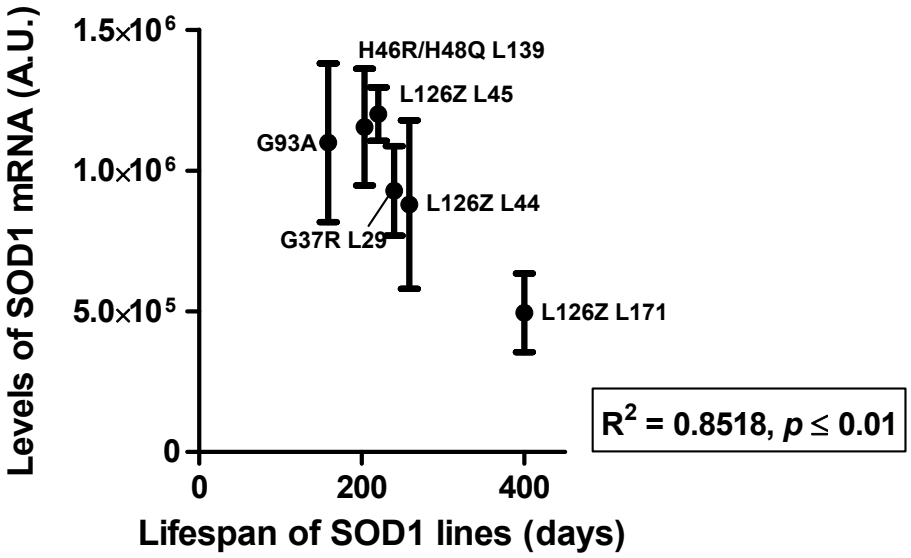
Supplemental Figure S1. Schematic representation of genomic SODMD. The SODMD DNA is mutated within each of the 5 exons (E). Red denotes single amino acid substitutions in the SOD1 protein that have been previously found in different human ALS patients. The rest of the single point mutations, in black, are experimental mutations. Orange circles indicate copper binding sites, while yellow circles indicate zinc binding sites. The first and last amino acid locations of each exon are also indicated in the figure.



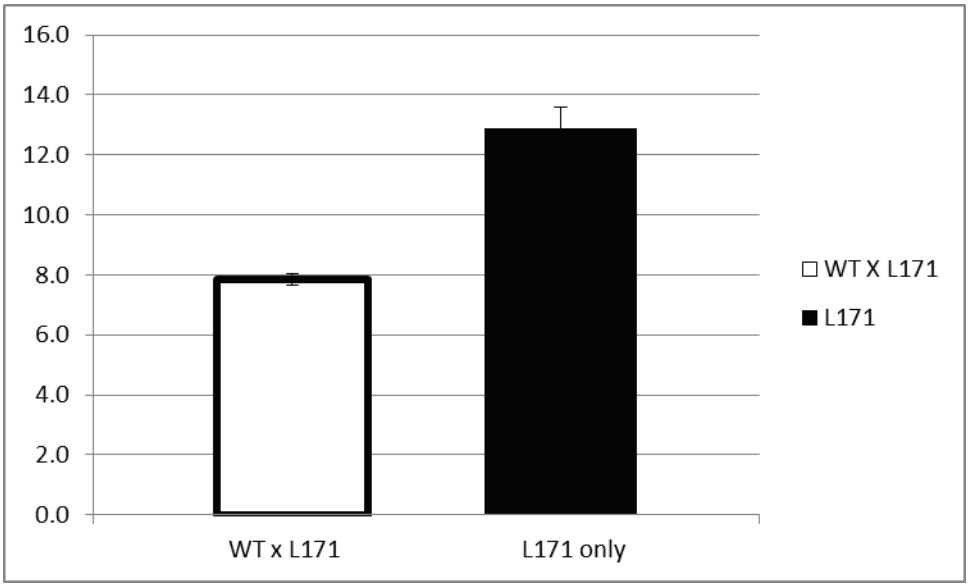
Supplemental Figure S2. Analyses of SODMD founder lines. A. Northern blot showing the two lines of SODMD mice with the best expression levels. The SODMD mRNA levels are compared to those of L126Z L171 which expresses SOD1 at very low levels. This blot was exposed for a considerable amount of time to allow visualization of the SODMD line I-32. **B.** Western blot showing SOD protein levels of SODMD higher expression lines using an antibody that recognizes both mouse (lower band, black arrowhead) and human (upper band, open arrowhead) SOD1. Note protein levels of SODMD line I-32 are considerably lower than line U-69. Thus we proceeded using the U-69 line for the rest of our studies.



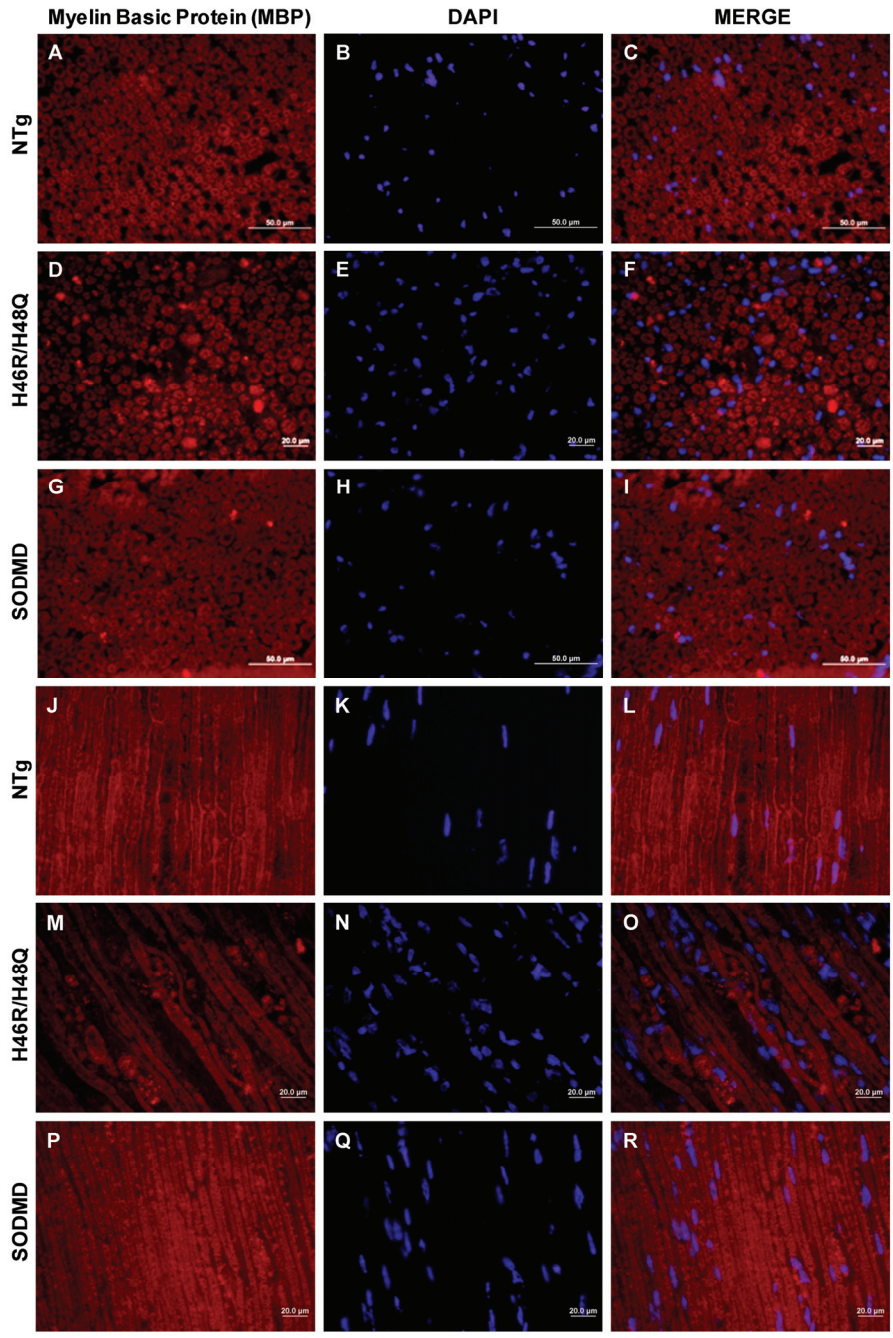
Supplemental Figure S3. Survival curves of different SOD1 lines. Survival times were collected from mutant SOD1 mice harvested over the course of 4 years. The number of animals for each genotypes were as follows; G93A (n=61), G46R/H48Q L139 (n=49), G37R L29 (n=58), L126Z L44 (n=21), L126Z L44 (n=69), L126Z L171 (n=10), MD U69 (n=37). For all lines of mutant mice, both male and female mice were included in the numbers. The survival data for WT L76 mice is based on the initial description of these mice (Wong et al., Neuron, 1995, Vol 14, p1105-1116).



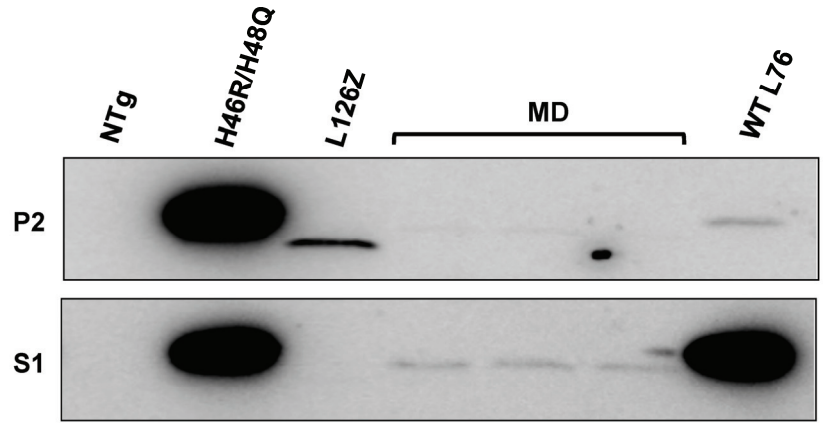
Supplemental Figure S4. The levels of SOD1 mRNA and lifespan of SOD1 mice statistically correlate. The levels of SOD1 mRNA were obtained from experiments presented in Figure 2a. Data on the lifespan of SOD1 were collected from a minimum of 10 mice per line of mice that were harvested at endstage, denoted by their characteristic hindlimb paralyses, along the course of 4 years.



Supplemental Figure S5. Comparison of age to paralysis for L126Z-Line 171 mice to mice co-expressing L126Z with WT SOD1. L126Z-Line 171 mice were mated to GurWT mice to generate doubly-transgenic animals. Mice that harbored both transgenes developed paralysis at a much younger age (avg 7.9 ± 0.4 mo; n = 4) than littermates that were transgenic only for L126Z-Line 171 (avg 12.9 ± 1.4 mo; n = 5). The y axis is age in months.

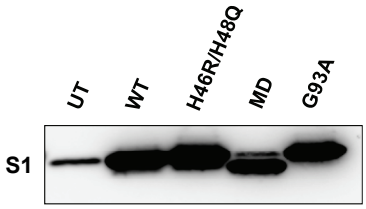


Supplemental Figure S6. Normal axonal architecture in aged SODMD mice. Transverse (**A-I**) or longitudinal (**J-R**) sciatic nerve sections were stained with myelin basic protein. DAPI nuclear stain was applied with secondary antibody incubations. All micrographs are at the same magnification and were taken with a 40x objective, bars = 50 (**A-C, G-I**) or 20 (**D-F, M-R**) µm. NTg = nontransgenic. All other genotypes are note on the figure. The images shown are representative of an analysis of at least 3 animals per genotype. SODMD and WT SOD1 mice were harvested at 2 years of age, and H46R/H48Q mice were harvested at symptomatic stages (~10 months).

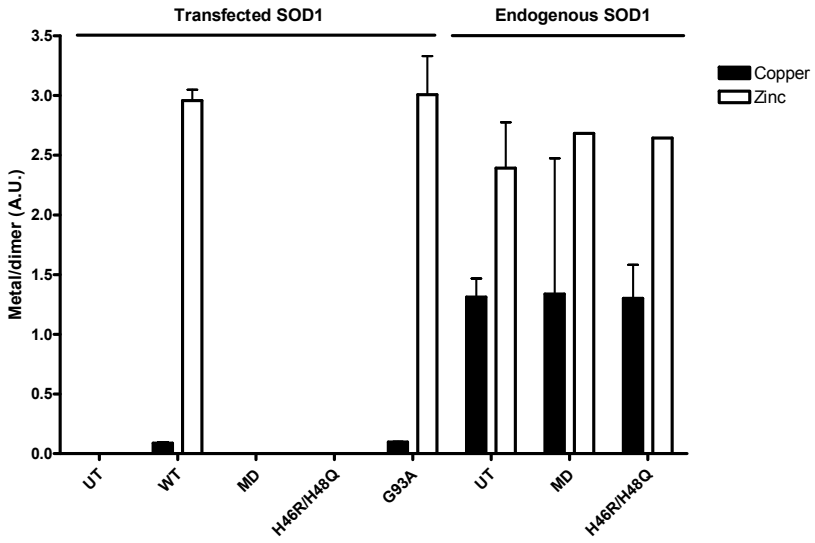


Supplemental Figure S7. SODMD protein does not accumulate as a detergent insoluble aggregate. **I**mmunoblot of detergent-insoluble (P2) and detergent-soluble (S1) protein fractions of mouse spinal cords, blotted with a human specific SOD1 antibody. NTg: non-transgenic mice. Note that L126Z protein although quite unstable in spinal cords (undetectable in S1), is aggregated at detectable levels while SODMD protein is not detectable at exposure levels that visualize the L126Z mutant in P2 fractions.

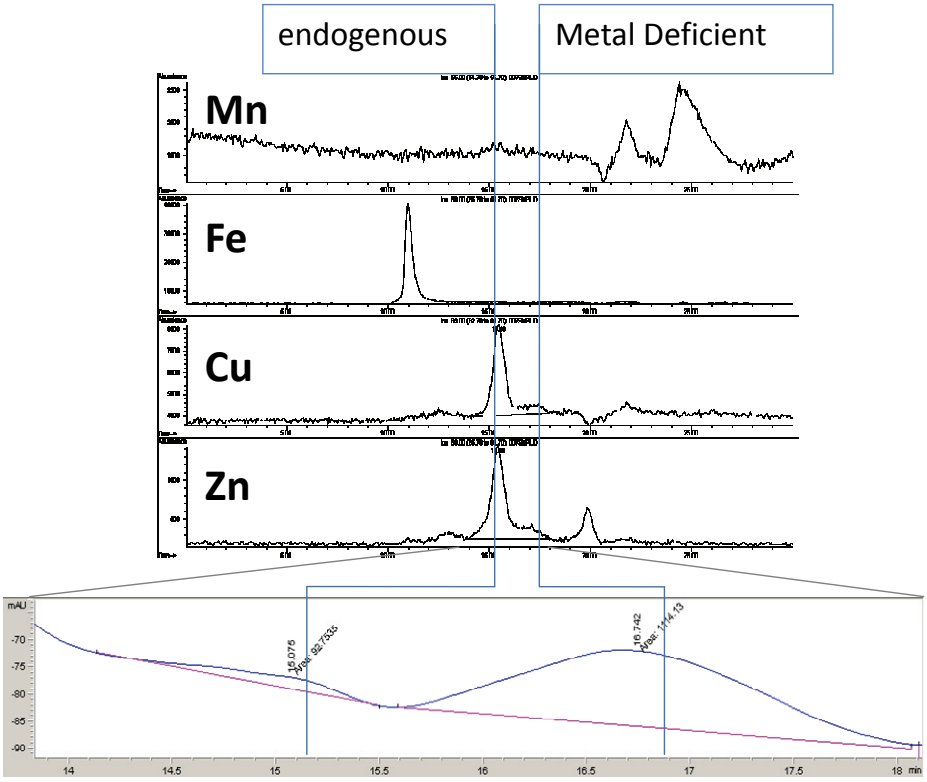
A


UT
WT
H46R/H48Q
MD
G93A
S1

B


Transfected SOD1
Endogenous SOD1
Copper
Zinc
Metal/dimer (A.U.)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
UT
WT
MD
H46R/H48Q
G93A
UT
MD
H46R/H48Q

C


endogenous
Metal Deficient
Mn
Fe
Cu
Zn
mAU
-70
-75
-80
-85
-90
15.075
16.742
14
14.5
15
15.5
16
16.5
17
17.5
18 min

Supplemental Figure S8. Metallation states of SOD1 expressed in HEK293FT cells. The metallation states were measured as described below. A) Example of SOD1 levels in S1 fractions from HEK293FT cells expressing the various SOD1 proteins. B) The left side of the graph shows the relative metallation state of the expressed SOD1 proteins. The graph on the right shows the metallation state of endogenous SOD1 in untransfected cells and in cells transfected with SODMD and SOD-H46R/H48Q. The H46R/H48Q hSOD1 and the metal deficient (MD) SOD1 did not bind detectable copper or zinc levels while G93A and WT bound very little copper but significantly higher levels of zinc. The metallation data from expressed WT, H46R/H48Q, MD, and G93A proteins were measured at least 3 times from 3 distinct transfection experiments. The metallation state of endogenous WT SOD1 in untransfected HEK293FT cells was also measured at least 3 times.

Because the G93A variant and overexpressed WT SOD1 do not separate from endogenous SOD1, we could not determine the metallation of endogenous SOD1 in these cultures. In cells expressing SODMD or SOD-H46R/H48Q, the metallation state of the endogenous SOD1 does not appear to change in two examples in which the WT protein could be definitively distinguished from the expressed protein. The values for Zn were measured only once, but the observed values match that of replicates of untransfected cells. The endogenous WT SOD1 in cells expressing H46R/H48Q and MD SOD1 were each measured only once, but the observed values match that of replicates in untransfected cells.

The metallation states were as follows:

Transfected SOD1				
	Cu/dimer	**Error**	**Zn/dimer**	**Error**
UT	n/a		n/a	
WT	0.0881	0.008681	2.9583	0.090629
MD	<0.0001		<0.1	
H46R/H48Q	<0.0001		<0.1	
G93A	0.0968	0.003615	3.0076	0.322463
Endogenous SOD1				
	Cu/dimer	**Error**	**Zn/dimer**	**Error**
UT	1.3114	0.157113	2.3929	0.383308
MD	1.3382	1.135312	2.6823	
H46R/H48Q	1.3019	0.280787	2.6448	

C) Representative chromatograms for SODMD. The endogenous mouse SOD1 elutes at 15.075 minutes and the SODMD variant elutes at 16.742 minutes off the HPLC. The corresponding metal profiles correspond to a metallated endogenous SOD1 as seen by the peaks in the Cu and Zn chromatograms but the SODMD mutant does not appear to have a significant amount of Mn, Fe, Cu or Zn associated with it. Note that the endogenous SOD1 does not co-elute with significant levels of Mn or Fe.

Supplementary Methods

Genotyping transgenic mice- All of the animals described in the main text and Supplemental Material were produced by in-house breeding. All studies included both male and female mice. Breeding stock (female F1 C57BL/6J x C3H/J) for the production of transgenic mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mouse tails were biopsied, removing approximately 0.6 cm of the distal tail. Collected tails were then digested and DNA extracted and analyzed for the presence of the transgene by PCR, as described previously (Prudencio, Durazo, Whitelegge, and Borchelt, *Hum. Mol. Genet. 2010, 19:4774-4789*). In order to distinguish human mutant SODMD from human WT SOD1 in double transgenic SODMD/WT SJL SOD1 mice, DNA from mouse tails positive for the human SOD1 transgene were re-amplified and the resulting DNA product was then purified using the PureLink PCR micro kit from Invitrogen (Carlsbad, CA), following the manufacturer protocol. The purified DNA fragments were then sent for automated sequence analysis together with the sequence primer SOD1-I3-AS (5'CTA TCG AAA GAC CTC AAG TAT AC-3').

Method for metal analysis - The level of Cu and Zn bound to soluble SOD1 isolated from cultured cells was determined using methods previously described by Lelie et al (Lelie HL, Liba A, Bourassa MW, Chattopadhyay M, Chan PK, Gralla EB, Miller LM, Borchelt DR. Valentine JS, Whitelegge JP. *J. Biol. Chem. 2011, 286:2795-2806*) with slight modification. Briefly, 40 µl cell culture supernatants (S1) were loaded onto an Agilent 1200 series HPLC column (SWX 2000 TOSOH Biosciences; resolvable range 5 kDa to 150 kDa), and the proteins were separated using a 30 minute isocratic gradient with a trace element free mobile phase of 25 mM potassium phosphate pH 6.7, 25 mM sodium chloride. The absorbance at 214 was used to monitor SOD1 peak elution and measure the protein concentration. The presence of SOD1 in the eluted peak was confirmed by immunoblotting. The eluent was then directed to the ICP-MS where copper, zinc, manganese, and iron concentrations were also measured real-time allowing for accurate copper and zinc concentration from the SOD1 peak. SOD1 metallation was determined by dividing SOD1 metal concentration by the SOD1 protein concentration.

Sciatic nerve extraction and immunostaining from SOD1 transgenic mice- Sciatic nerve was dissected and immersed in 3-methylbutane pre-chilled with liquid nitrogen for a few seconds, then immersed in liquid nitrogen. Tissue was then cut in sections of 5µm thickness, with a cryotome at -25ºC. For longitudinal sections, tissue freezing media (OCT, Electron Microscopy Sciences, Hatfield, PA) was applied on the base, without embedding the tissue. Flash Freezing spray (Thermo Fisher Scientific, Pittsburgh, PA) was then applied to the tissue prior cutting. For transverse sections the tissue was totally embedded in OCT before sectioning. To stain the sections, tissue was left to dry for about 1 h at room temperature before beginning staining procedure. Tissue was fixed in 4% paraformaldehyde for 10 minutes at room temperature, and permeabilized 5-10 minutes with 100% cold (-20ºC) methanol. Sections were rinsed 3 times with 1x PBS for 10 minutes each and blocked for 1 h at room temperature with 20% goat serum in 1x PBS. Myelin basic protein (MBP) antibody (Millipore – Chemicon, Billerica, MA) was used at 1:500 to stain slides overnight in 20% goat serum, 1x PBS at 4ºC. Tissue was then washed again 3 times and incubated with Alexa fluor goat anti-rabbit 594 nm (Invitrogen) in 20% goat serum

in PBS for 1 h. Finally, sections are washed 3 more times and coverslipped with DAPI (Invitrogen, Carlsbad, CA) at 1:2000.

Immunohistochemistry of spinal cord sections- Dissected spinal cords from mice that had been perfused with 4% paraformaldehyde were rinsed in distilled water for 30 minutes. The tissues were then incubated in the following series of solutions at room temperature with shaking unless otherwise indicated: 70% Ethanol for 15 minutes twice, 80% Ethanol for 15 minutes twice, 95% Ethanol for 15 minutes twice, 100% Ethanol four quick rinses, 100% Cedarwood oil for 15 minutes three times, pre-warmed Cedarwood oil to 37°C for 2 hours, Cedarwood/Methyl Salicylate for 40 minutes twice, Cedarwood/Methyl Salicylate overnight, Methyl Salicylate for 30 minutes three times, paraffin at 60°C for 30 minutes five times, fresh paraffin for 1 more hour. The tissue was embedded in 65°C paraffin. For sectioning, we produced 10 µm sections mounted on superfrost plus microscope slides (Fisher Scientific, Pittsburgh, PA).

Prior to staining, sections were incubated in a 60°C oven until the paraffin became clear. Sections were then were rehydrated and stained with one of the following described procedures. 1) Hematoxylin and eosin staining: slides were immersed into filtered Modified Mayer's Hematoxylin solution for 5 min, soaked in distilled water for about 2 min, immersed in eosin solution (0.1% eosin, 0.01% phloxine, 74% alcohol, 0,4% glacial acetic acid in water) for 2 min, and then water for 1 min before dehydrating and coverslipping. 2) Silver staining: slides were placed in 20% $AgNO_3$ in the dark for 20 min for impregnation followed by washing the slides in distilled water thoroughly and placing them into 20% $AgNO_3$ titrated with ammonium hydroxide, for 20 minutes in the dark. To develop slides we used titrated $AgNO_3$ with developer solution (30 ml distilled water, 5 ml of 37% formaldehyde, 1.5 g citric acid, and 1 drop of concentrated HNO_3) for 8-20 minutes. Then we washed slides with ammonium hydroxide in water and placed the slides in 5% $Na_2S_2O_3$ in water for 30 seconds, before washing again with distilled water, dehydrating, and coverslipping using permount. 3) GFAP-DAB staining: slides were pre-treated for 5 min in 0.3% H_2O_2, 10% Methanol in 1x PBS, then washed in 1x PBS for 10 minutes, 3 times. Blocking was done in 20% NGS, 0.1% Triton X-100 in 1x PBS for 45 min. GFAP antibody (Dako #Z0458, Carpinteria, CA) was used at 1:500 in 20% NGS, 0.1% Triton X-100 in 1x PBS with an overnight incubation. The next day slides were washed with 1x PBS for 10 min three times and incubated with biotinylated secondary anti-rabbit antibody (1:200) in 20% NGS, 0.1% Triton X-100 in 1x PBS for 1 hour. Slides were washed again in 1x PBS for 10 minutes once and incubated with ABC reagent for 30 min, then washed three times (10 min each) and developed in DAB (diaminobenzidine, Sigma-Aldrich, St Louis, MO) for about 5-20 minutes. Slides were washed again before counterstaining with hematoxylin for 5 min, dehydrating, and coverslipping.