Macrophage Migration Inhibitory Factor as a Chaperone Inhibiting Accumulation of Misfolded SOD1

Highlights
- A cytosolic chaperone inhibits ALS-causing mutant SOD1 binding to mitochondria and ER
- An unbiased screen identifies inhibition by MIF of ALS-causing mutant SOD1 misfolding
- Direct action of the ATP-independent chaperone activity of MIF reduces misfolded SOD1
- Elevation of MIF levels extends survival of mutant SOD1-expressing motor neurons

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In Brief
Israelson et al. identify MIF as a chaperone inhibiting misfolding of ALS-linked SOD1 mutant proteins. Elevating MIF suppresses mutant SOD1 association with intracellular organelles and extends survival of mutant SOD1-expressing motor neurons, supporting therapies to enhance intracellular MIF chaperone activity.
Macrophage Migration Inhibitory Factor as a Chaperone Inhibiting Accumulation of Misfolded SOD1

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SUMMARY

Mutations in superoxide dismutase (SOD1) cause amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by loss of motor neurons and accompanied by accumulation of misfolded SOD1 onto the cytoplasmic faces of intracellular organelles, including mitochondria and the endoplasmic reticulum (ER). Using inhibition of misfolded SOD1 deposition onto mitochondria as an assay, a chaperone activity abundant in nonneuronal tissues is now purified and identified to be the multifunctional macrophage migration inhibitory factor (MIF), whose activities include an ATP-independent protein folding chaperone. Purified MIF is shown to directly inhibit mutant SOD1 misfolding. Elevating MIF in neuronal cells suppresses accumulation of misfolded SOD1 and its association with mitochondria and the ER and extends survival of mutant SOD1-expressing motor neurons. Accumulated MIF protein is identified to be low in motor neurons, implicating correspondingly low chaperone activity as a component of vulnerability to mutant SOD1 misfolding and supporting therapies to enhance intracellular MIF chaperone activity.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive adult-onset neurodegenerative disorder characterized by the selective loss of upper and lower motor neurons. About 10% are inherited in a dominant manner (Da Cruz and Cleveland, 2011), with 20% of familial cases caused by mutation of cytoplasmic Cu/Zn superoxide dismutase (SOD1) (Rosen et al., 1993). The exact mechanism(s) responsible for motor neuron degeneration remains unsettled, albeit models for each of the nine most prominently proposed pathways include damage from misfolded, mutant SOD1 (Ilieva et al., 2009).

Multiple groups have identified that SOD1 mutants with divergent biochemical characteristics share a common property, with a proportion of the predominantly cytosolic SOD1 being localized to mitochondria (Israelson et al., 2010; Liu et al., 2004; Mattiazzi et al., 2002; Vande Velde et al., 2008) and/or the ER (Fujisawa et al., 2012; Nishitoh et al., 2008) but only in nervous system tissues in patient samples and rodent models. In particular, misfolded mutant SOD1 association with derlin-1, a component of the ER-associated degradation pathway, has been implicated in induction of ER stress from disrupted removal of misfolded proteins from the ER (Fujisawa et al., 2012; Nishitoh et al., 2008). Derlin-1 is bound by at least 132 of the ALS-linked SOD1 mutants, each of which exposes a derlin-1-binding domain buried in correctly folded SOD1 (Fujisawa et al., 2012).

Purification of mitochondria, including floation steps that eliminate protein-only aggregates, has demonstrated that mutant SOD1 deposition occurs on the cytoplasmic face of the outer membrane of spinal cord mitochondria (Liu et al., 2004; Vande Velde et al., 2008), accompanied by altered mitochondrial shape and distribution (Vande Velde et al., 2011). These findings were reinforced by demonstration (using sensitivity to proteolysis and immunoprecipitation with an antibody specific for misfolded SOD1) that misfolded forms of both dismutase active and inactive mutant SOD1 are deposited onto the cytoplasmic...
face of the outer membrane of spinal cord mitochondria (Vande Velde et al., 2008). One component directly bound by misfolded SOD1 is voltage-dependent anion channel-1 (VDAC1), with binding inhibiting its conductance of adenine nucleotides across the outer mitochondrial membrane (Israelson et al., 2010). Moreover, mutant SOD1 may also interact with other components of the mitochondrial outer membrane, including Bcl-2 (Pedrini et al., 2010) and the protein import machinery (Li et al., 2010), thereby altering the corresponding activities.

Recognizing that expression of SOD1 is ubiquitous but misfolded SOD1 is voltage-dependent anion channel-1 (VDAC1), with binding inhibiting its conductance of adenine nucleotides across the outer mitochondrial membrane (Israelson et al., 2010). Moreover, mutant SOD1 may also interact with other components of the mitochondrial outer membrane, including Bcl-2 (Pedrini et al., 2010) and the protein import machinery (Li et al., 2010), thereby altering the corresponding activities.

RESULTS

The Cytosol Determines Mutant SOD1 Association with Mitochondria and the ER

We previously reported that ALS-causing mutant SOD1 association with mitochondria was characterized by misfolded SOD1 binding to components, including VDAC1, on the outer mitochondrial membrane but was found for mitochondria isolated from spinal cord but not for those similarly purified from liver (Israelson et al., 2010). Consistent with this and other reports, immunoblot analysis of microsomes or mitochondria isolated from spinal cord homogenates (see schematic in Figure 1A) from rats expressing either of two ALS-linked mutations in SOD1 (SOD1G93A [Howland et al., 2002] and SOD1H46R [Nagai et al., 2001]) revealed that mutant SOD1 bound both to microsomal and mitochondrial membranes (Figure 1B). To determine whether this selective association of mutant SOD1 with spinal cord mitochondria was mediated by the spinal cord mitochondria or
the corresponding cytosol, we isolated normal, nontransgenic mitochondria from spinal cord and liver. Those mitochondria were then incubated (Figure 1C) with cytosolic extracts prepared (as in Figure 1A) from spinal cords or livers of rats expressing dismutase active (SOD1 G93A) or inactive (SOD1 H46R) mutants. The mitochondria were reisolated and analyzed by immunoblotting for mutant SOD1. Although the liver and spinal cord cytosols contained comparable levels of mutant human SOD1 (Figure 1C, bottom), none of the mutant SOD1 in liver cytosol bound to liver- or spinal cord-derived nontransgenic mitochondria. In contrast, incubation with spinal cord cytosols from SOD1 G93A- or SOD1 H46R-expressing rats did yield association of a proportion of both mutant SOD1s with wild-type mitochondria. For both dismutase active (SOD1 G93A) and inactive (SOD1 H46R) mutants, binding was independent of the tissue from which the mitochondria were isolated (Figure 1C). Thus, the tissue origin of the cytosol, not of the mitochondrion, determines whether mutant SOD1 in those cytosols binds to mitochondria.

Identification of MIF as an Inhibitor of Mutant SOD1 Association with Mitochondria

To identify the activity responsible for blocking association of mutant SOD1 with mitochondria, liver cytosol was incubated with proteinase K and the resultant peptides and other low-molecular-weight species below 10 kDa were removed by centrifugal filtration. Remaining proteins and other macromolecules were then fractionated by gel permeation chromatography (see schematic in Figure 2A). Finally, fractions were assayed for the ability to inhibit recombinant SOD1 G85R binding to nontransgenic mitochondria. Activity eluted broadly with apparent molecular weights between 12 and 45 kDa. Proteins in the fractions with activity (fractions 23–41; Figure 2C) were visualized by PAGE (Figure S1). Remarkably, although the inhibitory activity was heat labile (consistent with a protein activity), it was not inactivated by degradation of most proteins by incubation of the liver cytosol with proteinase K (Figure 1D, lane 3).

Next, we investigated whether there was a factor(s) in spinal cord cytosol that induced association of SOD1 with normal mitochondria or, in contrast, whether some factor in the liver prevented binding. To do this, recombinant SOD1 G85R was incubated with mitochondria purified from liver of nontransgenic rats in the presence of SOD1 G93A spinal cord or liver extract. Whereas spinal cord cytosol did not affect SOD1 G85R association with mitochondria (Figure 1D, lane 5), added liver cytosol inhibited it (Figure 1D, compare lanes 1 and 2). This inhibitory activity was not affected by inhibitors of the Hsp70 and Hsp90 protein chaperones and was independent of calcium (Figure S1).
and Coomassie staining (Figure 2B). The most slowly eluting fraction with activity (fraction 41) had only a handful of observable polypeptides (Figure 2B). We therefore used mass spectrometry to analyze its protein content. Although the sensitivity of mass spectrometry frequently identifies many proteins present in such assays, remarkably, however, only seven proteins were detected (Figure 2D). One of these, profilin-1, an actin-binding protein linked to regulation of actin polymerization, was notable, as mutations in it are causative of a small proportion of inherited ALS (Wu et al., 2012). Nevertheless, immunoblotting was used to determine that profilin-1 was absent from many fractions (23–30) that retained inhibitory activity (Figure 2E).

Similar immunoblotting with antibodies to each of the six remaining proteins determined that only one cofractionated with the inhibitory activity (Figure 2E). This was the 12-kDa macrophage migration inhibition factor, which has previously been implicated in divergent functional roles: as a protein folding chaperone (Cherepkova et al., 2006), thiol-oxidoreductase protein (Kleemann et al., 1998), and secreted cytokine with an important role in innate immunity (Calandra and Roger, 2003; Lolis and Bucala, 2003). MIF eluted broadly in fractions spanning from 12 to 45 kDa apparent molecular weights, just as did the activity for inhibiting mutant SOD1 binding to mitochondria. The broad elution of MIF was not because of poor resolution of the column, as other proteins (including alcohol dehydrogenase, profilin-1, and SOD1 itself) fractionated much more sharply. Rather, the broad elution was fully consistent with MIF’s known ability to form dimers and trimers (Mischke et al., 1998; Philo et al., 2004) through an exposed hydrophobic face as a part of its ATP-independent activity as a protein folding chaperone (Cherepkova et al., 2006). Interestingly, MIF protein levels do not change within the spinal cord during disease course in SOD1 mutant mice (Figure S2).

**Dose-Dependent Inhibition by MIF of Mutant SOD1 Association with Mitochondria**

As a direct test of whether MIF inhibits association of mutant SOD1 with mitochondria, purified mutant or wild-type SOD1 (Figure 3A, bottom right) was added to normal liver mitochondria in the presence or absence of purified MIF (see schematic in Figure 3A). After recovering the mitochondria by centrifugation, immunoblotting was used to reveal that a proportion of SOD1G93A and SOD1G85R, but not SOD1WT, bound to nontransgenic mitochondria in a dose-dependent manner (Figure S3) in the absence of MIF (Figure 3B). Mutant SOD1 binding was inhibited in a dose-dependent manner by coincubation with MIF, with a 1:80 molar ratio of MIF to SOD1 (50 ng of MIF and 4 μg of mutant SOD1), sufficient to strongly diminish (by >90%) binding of mutant SOD1 to mitochondria (Figure 3C). Importantly, about 5%–10% of recombinant mutant SOD1 bound to mitochondria in these assays (Figure 3C), a percentage of misfolded SOD1 similar to that measured in immunoprecipitates of the initial recombinant SOD1 preparation when using B8H10 antibodies that bind misfolded SOD1 (Figure S5).

We exploited the known proteinase K resistance or sensitivity, respectively, of correctly folded and misfolded SOD1G93A (Borchelt et al., 1994; Ratovitski et al., 1999) to test whether the mutant SOD1 that associates with mitochondria represents the initially misfolded fraction. We initially verified that most of the dismutase active SOD1G93A is protease resistant; in contrast, most of the inactive SOD1G85R was sensitive (Figure 3E). SOD1G93A was preincubated with proteinase K, the protease was then inactivated by addition of PMSF and the mixture was incubated with normal liver mitochondria, and finally mitochondria along with any bound mutant SOD1 were recovered by centrifugation. Immunoblotting for human SOD1 demonstrated that SOD1G93A association with mitochondria was completely eliminated by proteinase K preincubation, confirming that the protease-sensitive, misfolded fraction is the one that associates with mitochondria (Figure 3D).

Inhibition of misfolded SOD1 association with mitochondria was specific to MIF, as other chaperones tested, including Hsp27, Hsc70, aB-crystallin (previously reported to interact with mutant and misfolded SOD1 and modulate its aggregation; Karch and Borchelt, 2010; Krishnan et al., 2008; Wang et al., 2005, 2009; Yerbury et al., 2013; Zetterström et al., 2011b), cyclophilin-A (one of our seven candidate proteins and previously proposed to act as a chaperone; Freskgård et al., 1992), or glutathione peroxidase (a protein with thiol-oxidoreductase activity, an activity that has been proposed to be crucial for the association of mutant SOD1 proteins with mitochondria; Cozzolino et al., 2008; Ferri et al., 2006), had no effect on mutant SOD1 association with normal mitochondria (Figures 3F and 3G).

**MIF Suppresses Misfolded Mutant SOD1 Association with ER and Mitochondrial Membranes in Motor Neuron-like Cells**

We next tested whether MIF inhibited association of mutant SOD1 not only with mitochondria but also with other intracellular membranes in motor neuron-like (NSC-34) cells expressing ALS-linked SOD1 mutants (see schematic in Figure 4A). EGFP-tagged SOD1G93A or SOD1G85R was expressed by DNA transfection, along with lower or higher amounts of MIF. Accumulated levels were validated by immunoblotting for each protein (Figure 4B, bottom), and the association of mutant SOD1 with ER membranes was determined after isolation of microsomes. Both dismutase active (SOD1G93A) and dismutase inactive (SOD1G85R) mutants bound to ER membranes in the presence of the low endogenous levels of MIF (Figure 4B, lanes 1 and 4), but binding in both cases was reduced in a dose-dependent manner when MIF levels were elevated (Figure 4B). MIF also inhibited mutant SOD1 association with mitochondria (Figure 4C).

We next tested whether MIF also inhibited accumulation of misfolded SOD1 in a dose-dependent manner in the same motor neuron-like cells expressing ALS-linked SOD1 mutants (see schematic in Figure 4D). In the absence of transfected MIF, misfolded SOD1 accumulated and could be detected by immunoprecipitation with the B8H10 antibody, which does not recognize wild-type SOD1 but does recognize a wide spectrum of misfolded SOD1 mutants (Gros-Louis et al., 2010) including SOD1G93A and SOD1G85R (Figure 4E, lanes 2 and 3). Transfection to simultaneously express MIF along with either SOD1G93A or SOD1G85R reduced the level of misfolded SOD1 (Figure 4E, lanes 4 and 5), without affecting the overall level of accumulated mutant SOD1. Similar results were observed by expressing...
MIF in the presence of untagged versions of SOD1 \(^{G93A}\) or SOD1 \(^{G85R}\) in human SH-SY5Y neuroblastoma cells (Figure S4). The Thiol-Oxidoreductase Activity of MIF Is Not Necessary for Suppressing Misfolded SOD1

One of MIF's previously documented enzymatic roles is as a thiol-oxidoreductase (Kleemann et al., 1998). SOD1 monomers lacking the C57-C146 disulfide bond represent the major portion of misfolded SOD1 (Zetterström et al., 2013), thus suggesting that a thiol-oxidoreductase activity may play an important role in misfolded protein formation. To determine whether the action of MIF in suppressing accumulation of misfolded SOD1 required this activity, cells were transfected with a point mutant of MIF (MIF\(^{C60S}\)) previously shown to completely lack oxidoreductase activity (Kleemann et al., 1998). Coexpression of MIF\(^{C60S}\) in NSC-34 cells with either SOD1\(^{G93A}\) or SOD1\(^{G85R}\) inhibited the accumulation of misfolded SOD1 in a dose-dependent manner (Figure 4F), consistent with an in vivo ability to suppress mutant SOD1 accumulation onto microsomal (Figure 4B) or mitochondrial membranes (Figure 4C). Misfolding of SOD1\(^{G93A}\) which can acquire normal folding (as indicated by dimerization, protease resistance, and high specific activity as a superoxide

Figure 3. Recombinant MIF Inhibits Association of Mutant SOD1 with Mitochondria in a Dose-Dependent Manner

(A) Schematic of a protocol to test whether purified recombinant MIF inhibits association of recombinant SOD1\(^{G93A}\) or SOD1\(^{G85R}\) with normal liver mitochondria. Lower right: Coomassie-stained SDS-polyacrylamide gel analysis of recombinant SOD1 and MIF. (B) Immunoblotting of mitochondria recovered from the protocol in (A) and assayed for MIF-dependent inhibition of association of either SOD1\(^{G93A}\) or SOD1\(^{G85R}\) when coincubated with nontransgenic liver mitochondria. Immunoblotting for VDAC1 was used to verify the amount of mitochondria added/recovered. Bar values represent the means ± SEM of three independent experiments. (C) Immunoblot assay as in (A) for dose-dependent inhibition by recombinant MIF of mutant SOD1\(^{G93A}\) binding to nontransgenic liver mitochondria. Immunoblotting for VDAC1 was used to determine mitochondrial recovery. Immunoblotting for MIF was used to determine the absence of MIF in the mitochondrial fraction and the increased levels of MIF in the supernatant that were used. (D) Immunoblotting of mitochondria recovered from the protocol in (A) and assayed for proteinase K-dependent inhibition of association of SOD1\(^{G85R}\) with nontransgenic liver mitochondria. Immunoblotting for VDAC1 was used to verify the amount of mitochondria added/recovered. (E) Immunoblotting was used to determine the effect of proteinase K on recombinant SOD1\(^{G93A}\) or SOD1\(^{G85R}\). (F and G) Assay patterned after the protocol in (A) testing for whether purified (F) MIF, Hsp27, cyclophilin-A (Cyp-A), or glutathione peroxidase (Gpx) or (G) Hsc70 or a-crystallin inhibits SOD1\(^{G85R}\) association with nontransgenic liver mitochondria. Some lanes were not loaded in the same order as shown in the figure.
MIF Directly Suppresses the Accumulation of Misfolded SOD1 In Vitro

To determine whether MIF directly inhibits the accumulation of misfolded SOD1, immunoprecipitation was performed (see schematic in Figure 5A) with a previously reported conformation-specific antibody (DSE2) that selectively recognizes misfolded, but not correctly folded, SOD1 (Israelson et al., 2010; Vande Velde et al., 2008). As expected, a proportion of SOD1G93A and SOD1G85R, but not SOD1WT, was immunoprecipitated with the DSE2 antibody (Figure 5B, lanes 3 and 5). Incubation of the mutant SOD1 with MIF nearly eliminated misfolded SOD1G93A that could be immunoprecipitated by DSE2 (Figure 5B, lane 3 versus 4). MIF also substantially inhibited accumulation of misfolded SOD1G85R (Figure 5B, lanes 5 and 6). Remarkably, a similar level of inhibition was observed when the SOD1G85R mutant was incubated with a liver cytosolic fraction that contained endogenous MIF accumulated to a similar level (Figure 5B, lane 7).

Next, purified SOD1G93A was depleted of initially misfolded SOD1 by immunoprecipitation with the B8H10 antibody (Figure 5D, lane 2), the unbound fraction free of misfolded SOD1 was incubated at 37°C in the absence (lane 3) or presence (lane 4) of MIF, and finally newly generated misfolded SOD1 was detected with a second round of immunoprecipitation with the B8H10 antibody. Remarkably, MIF suppressed formation of newly misfolded SOD1, as revealed by immunoblotting the final immunoprecipitate (Figure 5D, lane 4). Suppression was specific for MIF, because neither a β-crystallin nor Hsc70 (added at comparable concentrations) inhibited formation of newly misfolded SOD1 (Figure S6).

Possible direct binding of MIF to mutant SOD1 was tested by covalently labeling purified MIF with a green fluorescent dye (at a ratio of one labeled lysine residue per one MIF molecule) and incubating it with increasing concentrations of purified SOD1G93A (2.4 nM to 80 μM). Binding of the two was quantified using microscale thermophoresis, a sensitive protein-protein interaction assay (Wienken et al., 2010) that measures changes in the thermal migration behavior of particles in a temperature gradient as influenced by a binding partner. By plotting the percentage of change in normalized fluorescence as a function of SOD1G93A concentration (Wienken et al., 2010), curve fitting to the data points produced an excellent fit with a calculated dissociation constant (Kd) of 367 nM for interaction of mutant SOD1G93A and MIF (Figure 5E).

A Low Level of Endogenous MIF within Motor Neuron Perikarya

Immunostaining for MIF in nontransgenic rat spinal cord revealed it to be accumulated widely, with the striking exception of almost complete absence within choline acetyltransferase (ChAT)-positive motor neuron cell bodies (Figure 6A). In contrast, MIF was readily detected in astrocytes (Figure 6B). Although no change was apparent in overall MIF protein levels within the spinal cord during disease course (Figure S2), immunostaining revealed increased MIF in reactive astrocytes just after symptomatic onset (i.e., 1 week after the first fibrillation) in a SOD1G93A rat (Figure 6D; Figure S7). Nevertheless, MIF protein remained undetectable in motor neurons, and this low level of intraparenchymal MIF was accompanied by accumulation of misfolded SOD1 (recognized by the B8H10 antibody) (Figures 6C and 6D; arrows in Figures 6E; Figure S7).

To determine whether the absence of accumulated MIF in motor neurons resulted from a low level (or absence) of synthesis of MIF in those neurons, we exploited bacTRAP reporter mouse lines (Doyle et al., 2008; Heiman et al., 2008) (see schematic in Figure 7A) that express EGFP-tagged ribosomal protein L10a (Rpl10a) driven by cell type-specific transgene promoters in motor neurons (Chat-bacTRAP), astrocytes (Aidh1fl1-bacTRAP), or oligodendrocytes (Cnp1-bacTRAP) (Doyle et al., 2008; Heiman et al., 2008). Analysis of affinity-isolated, actively translating, polyribosome-associated mRNAs from each cell type (Figure 7B) revealed that translating MIF mRNA was found in all three cell types, with motor neurons accumulating the highest level (Figure 7C). Thus, MIF is synthesized actively by motor neurons, and its low accumulation in motor neuron cell bodies (Figure 6) must be the result of rapid clearance (secretion, degradation, or transport) from the perikarya of those neurons.

Increased MIF Enhances Survival of Mutant SOD1-Expressing Motor Neurons

To test whether increased synthesis and accumulation of MIF could be protective of mutant SOD1-expressing motor neurons, motor neurons were isolated by fluorescence-activated cell sorting (FACS) for expression of an HB9-promoted GFP motor neuron reporter gene following differentiation from nontransgenic (non-Tg) mouse embryonic stem cells or induced pluripotent stem cells (iPSCs) derived from mice expressing human wild-type (SOD1WT) or mutant (SOD1G93A) SOD1 transgenes (see schematic in Figure 8A). MIF synthesis was elevated by transduction with a lentivirus (Lv) encoding MIF and red fluorescent protein (RFP) (the latter translated from an internal ribosomal entry site in the MIF 3’ UTR). Beginning day 3 post-transduction, intraneuronal levels of MIF (detected by indirect immunofluorescence with MIF antibody) were elevated relative to motor neurons transduced with RFP alone (Figure 8B). The contrast between the failure of endogenously synthesized MIF to accumulate in mature motor neurons (Figure 6) and its accumulation via lentiviral transduction in cultured motor neurons (Figure 8) can be explained in two ways. Elevated MIF synthesis in cultured motor neurons may saturate degradation or secretion machineries. Alternatively, the degradation/secretion of endogenous MIF in mature motor neurons may reflect a non-cell-autonomous influence, especially on secretion, exercised by the partner glial cells, which are missing in our pure neuronal cultures.

Direct live-cell imaging (Figure 8C) revealed that, compared to nontransgenic or SOD1WT motor neurons, only one-fifth as many mutant SOD1G93A-expressing motor neurons survived the first 2 days of culture prior to MIF synthesis (Figures 8D–8F). MIF expression, however, significantly attenuated this accelerated motor neuron death at subsequent time points (Figure 8F).
Figure 4. MIF Inhibits the Association of Misfolded Mutant SOD1 with Intracellular Membranes in a Cell-Culture Model of ALS

(A) Schematic of a protocol for assaying MIF-dependent inhibition of mutant SOD1 association in vivo with microsomes or mitochondria following expression of MIF and SOD1G93A or SOD1G85R in motor neuron-like NSC-34 cells.

(legend continued on next page)
Similarly, increased MIF significantly increased neurite length of the surviving SOD1<sup>G93A</sup> motor neurons (Figure 8G).

**DISCUSSION**

Among the important unsolved questions in the disease mechanism from ubiquitous expression of mutant SOD1 is what determines the selective, age-dependent motor neuronal degeneration that is accompanied by mutant SOD1 misfolding and its association with mitochondria and the ER. We have now determined that both dismutase active and inactive SOD1 mutant association with such organelles can be suppressed by cytosolic MIF acting cata lytically to inhibit misfolded SOD1 accumulation and its association with mitochond ria and the ER. Furthermore, increased MIF, which normally accumulates only to low levels within the cell bodies of motor neurons, suppresses misfolded SOD1 accumulation and extends mutant SOD1-expressing motor neuron survival in cell culture. The low level of MIF accumulated within motor neurons correlates with accumulation of misfolded SOD1 species and their association with different intracellular organelles within those neurons.

Despite its small size (12 kDa), MIF has previously been implicated in both extracellular and intracellular roles. MIF was one of the first cytokines to be described (George and Vaughan, 1962), with its action in the immune response upstream of tumor necrosis factor-α, interleukin-1β, interferon-γ, and other effector cytokines (Calandra and Roger, 2003). MIF is synthesized as a cytoplasmic protein (a point we have validated after removal of endogenous SOD1 binding to intracellular targets. From these collective efforts, we now propose that low MIF chaperone-like activity in alpha motor neurons plays a pivotal role in misfolded SOD1 accumulation and subsequent toxicity.

Of relevance to MIF’s extracellular activities, mutant SOD1 synthesized by microglia drives rapid disease progression (Beers et al., 2006; Boillée et al., 2006). Additionally, immunodeficiency shortened mutant SOD1 mouse lifespan (Beers et al., 2008; Chiu et al., 2008) whereas, conversely, passive transfer of activated T regulatory cells to immunocompetent mice modestly extended survival (Beers et al., 2011). We note that one of the manifestations of immune deficits in ALS could be a malfunction of MIF, thereby leading to accumulation of soluble misfolded SOD1 and binding to intracellular targets. Indeed, recognizing the potential for cell-to-cell spread of misfolded SOD1 as a means of disease propagation (Grad and Cashman, 2014; Grad et al., 2011; Münch et al., 2011), chaperone activity by extracellular MIF may act to limit such spreading.

**(B and C)** Immunoblot for mutant SOD1 associated with (B) microsomes or (C) mitochondria purified from NSC-34 cells transfected to express mutant SOD1<sup>G93A</sup> or SOD1<sup>G93H</sup> and low or high levels of MIF. Parallel immunoblotting for calnexin or VDAC1 was used to verify comparable recovery of microsomes or mitochondria, respectively. Similar immunoblot analyses of the initial cytosols were used to determine initial accumulated levels of MIF and mutant SOD1. Bar values represent the means ± SEM of three independent experiments.

**(D)** Schematic of protocol to test whether expression of MIF, with or without its thiol-oxidoreductase activity, can act in vivo to suppress accumulation of misfolded mutant SOD1 within NSC-34 cells.

**(E and F)** MIF was expressed by transient transfection in NSC-34 motor neuron-like cells also transfected to express wild-type or mutant human SOD1. Misfolded SOD1 was detected by immunoblotting of immunoprecipitates produced with the B8H10 antibody, which recognizes epitopes within exon 3 that are buried in correctly folded SOD1. IP, immunoprecipitation.

**(G)** Expression of wild-type MIF.

**(H)** Expression of MIF<sup>C60S</sup> lacking thiol-oxidoreductase activity. MIF, endogenous SOD1, and EGFP-tagged wild-type or mutant human SOD1 levels were determined by immunoblotting in the unbound or initial cytosol fractions.
Finally, misfolded SOD1 has been reported by several groups to accumulate in motor neurons or glia in sporadic ALS (Bosco et al., 2010; Forsberg et al., 2010, 2011; Grad et al., 2014; Guarbeschi et al., 2012; Kabashi et al., 2007; Pokrisshevsky et al., 2012; Zetterström et al., 2011a), although other efforts have reached opposite conclusions (Ayers et al., 2014; Brotherton et al., 2012; Kerman et al., 2010; Liu et al., 2009). Astrocytes generated from neuronal progenitor cells (NPCs) isolated from spinal cords of sporadic ALS patients have been found to be toxic to cocultured motor neurons (Haidet-Phillips et al., 2011), similar to what had been seen previously for astrocytes expressing mutant SOD1 (Di Giorgio et al., 2007; Marchetto et al., 2008; Nagai et al., 2007). Surprisingly, reducing wild-type SOD1 with small hairpin RNA significantly reduced toxicity to motor neurons of most of the “sporadic” ALS astrocytes (Haidet-Phillips et al., 2011), a finding directly disputed by others who concluded that wild-type SOD1 plays no role in the toxicity of sporadic ALS-derived astrocytes to cocultured motor neurons (Re et al., 2014).

The controversy notwithstanding, misfolded protein accumulation is central to essentially all instances of inherited and sporadic ALS. Identification of MIF as an intracellular chaperone that stimulates folding/refolding of misfolded SOD1 suggests a new avenue for therapy development in ALS through increasing intracellular MIF levels. Combined with recognition that extracellular MIF is an inducer of metalloproteinase 9 (MMP9) (Yu et al., 2007), a component contributing to the selectivity of motor neuron vulnerability to SOD1 mutant-mediated death (Kaplan et al., 2014), this finding underscores how approaches to increasing intracellular MIF (e.g., by reducing its clearance from motor neurons) and/or to inhibiting its induction of MMP9 (e.g., with drugs that block MIF’s interaction with its known receptor [CD74; Bai et al., 2012]) could be attractive therapeutic strategies.

Figure 5. MIF Suppresses Misfolded Mutant SOD1 Accumulation by Directly Acting on It
(A) Protocol to determine whether purified recombinant MIF suppresses accumulation of misfolded SOD1 detectable with the DSE2 antibody, which recognizes an epitope in the electrostatic loop of hSOD1 (between residues 125 and 142) that is buried in correctly folded SOD1.
(B) Accumulation of misfolded SOD1 was determined by immunoblotting of immunoprecipitates with the DSE2 antibody after incubation of recombinant hSOD1 wild-type, hSOD1G93A, or hSOD1G85R (4 μg) in the absence or presence of recombinant MIF (100 ng). Immunoblotting was used to determine MIF levels remaining in the unbound fraction of each immunoprecipitation assay.
(C) Protocol to determine whether purified recombinant MIF suppresses accumulation of newly formed, misfolded SOD1 detectable with the B8H10 antibody for misfolded SOD1.
(D) Misfolded SOD1 determined by immunoblotting of immunoprecipitates of recombinant hSOD1G93A with the B8H10 antibody (lane 1). The unbound fraction was subjected to a second immunoprecipitation performed immediately (lane 2) or after a 5-hr incubation in the (lane 3) absence or (lane 4) presence of recombinant MIF. Immunoblotting was also used to determine MIF levels remaining in the unbound fraction of each immunoprecipitation assay.
(E) Assay of MIF binding to mutant SOD1. Purified MIF (200 nM) was fluorescently labeled and incubated for 20 min at room temperature with increasing concentrations (from 2.4 nM to 80 μM) of SOD1G93A. Closed diamonds indicate binding of MIF to SOD1G93A determined by microscale thermophoresis assay. The smooth curve represents the predicted binding of MIF to mutant SOD1 calculated by curve fitting with a Kd of 367 nM (see Jerabek-Willemsen et al., 2011; Parker and Newstead, 2014; Wienken et al., 2010).
Figure 6. A Low Level of MIF in Motor Neuron Cell Bodies Is Accompanied by Diffuse Accumulation of Misfolded SOD1 within Those Cell Bodies Just after Disease Onset in SOD1<sup>G93A</sup> Rats

(A) Accumulation of MIF in lumbar spinal cord of a nontransgenic rat assayed by indirect immunofluorescence with antibodies to MIF along with simultaneous identification of motor neurons (with antibodies to ChAT) and misfolded SOD1 (with the B8H10 antibody).

(B) An analogous assay as in (A) but with astrocytes identified with an antibody to glial fibrillary acidic protein (GFAP).

(C) Accumulation of MIF in lumbar spinal cord of an early symptomatic SOD1<sup>G93A</sup> rat, 1 week after the first spontaneous muscle fibrillation, along with simultaneous identification of motor neurons (identified by morphology, position, and antibodies to NeuN) and misfolded SOD1 (with the B8H10 antibody).

(D) An analogous assay as in (C) but with astrocytes identified with an antibody to GFAP. The scale bar represents 50 µm.

(E) Higher magnification of the insets outlined in (D). The arrows highlight a motor neuron lacking MIF immunostaining, and asterisks mark GFAP-positive astrocytes containing MIF.
EXPERIMENTAL PROCEDURES

Transgenic Animals
Transgenic rats expressing hSOD1<sup>WT</sup> (Chan et al., 1998), hSOD1<sup>G93A</sup> (Howard et al., 2002), and hSOD1<sup>H46R</sup> (Nagai et al., 2001) were as originally described. Transgenic mice expressing mutant SOD1<sup>G93A</sup> (line 148), SOD1<sup>H46R</sup> (line 42), or SOD1<sup>G85R</sup> were maintained by standard protocols in the D.W.C. laboratory. bacTRAP transgenic mouse lines: Chat<sup>-bacTRAP</sup> line expresses EGFP-tagged ribosome protein Rpl10a only within motor neurons. The Aldh1l1<sup>-bacTRAP</sup> line expresses the same EGFP-tagged Rpl10a in astrocytes, whereas the Cnp1<sup>-bacTRAP</sup> line expresses in mature oligodendrocytes (Doyle et al., 2008; Heiman et al., 2008). All animal procedures were consistent with the requirements of the Animal Care and Use Committees of the University of California and Ben-Gurion University of the Negev.

Mutant SOD1 Binding to Mitochondria
Spinal cord or liver cytosolic fractions (200 μg) from hSOD1<sup>G93A</sup> (C24 90-d-old) or hSOD1<sup>H46R</sup> (C24 200-d-old) symptomatic female rats or recombinant hSOD1<sup>WT</sup>, hSOD1<sup>G93A</sup>, or hSOD1<sup>G85R</sup> proteins (4 μg) were incubated with spinal cord or liver mitochondria (50 μg) isolated from nontransgenic rats for 30 min at 37°C in the presence or absence of recombinant mouse MIF (R&D Systems) at the indicated concentration. Where indicated, recombinant mutant SOD1 was incubated with proteinase K (100 μg/ml) for 15 min. The reaction was stopped by addition of 10 mM PMSF followed by incubation on ice for 10 min. Then, the mitochondrial fraction was recovered by centrifugation at 12,000 × g for 10 min at 4°C and washed twice with mitochondrial buffer. The pellet was resuspended with sample buffer and run on SDS-PAGE.

iPSC Generation
Neuronal progenitor cells expressing the motor neuron HB9::GFP reporter obtained from SOD1<sup>WT</sup> and SOD1<sup>G93A</sup> mice were converted to iPSCs. As previously described, retrovirus encoding OCT3/4 and KLF4 was sufficient to generate iPSC clones (Hester et al., 2009; Kim et al., 2008). Twenty viral particles per cell were needed to efficiently reprogram the cells. Cells were cultured in the presence of NPC media for 4 days followed by a change to mouse embryonic stem cell (mESC) media with DMEM (Millipore), supplemented with ES fetal bovine serum (18%; Invitrogen), L-glutamine (2 mM; Invitrogen), nonessential amino acids (1/3; Millipore), antibiotic-antimycotic (1%; Invitrogen), 2-mercaptoethanol (114 μM; Sigma), and recombinant LIF (100 U/ml; Millipore). iPSC clones were morphologically similar to mouse ESCs (HBG3 cells; Thomas Jessell, Columbia University) and were obtained within 2 weeks. A wide panel of markers was used to compare ESCs with the newly generated iPSC lines and found no significant difference in their expression between cell lines.

Mouse Motor Neuron Differentiation
Mouse ESCs or iPSCs expressing HB9::GFP reporter were cultured on top of inactivated mouse fibroblasts (Millipore). Motor neuron differentiation was induced by plating 1–2 × 10<sup>6</sup> mES cells per 10-cm dish in the presence of 2 μM retinoic acid (Sigma-Aldrich) and 2 μM purmorphamine (Calbiochem). After 5 days of differentiation, embryonic bodies were dissociated and sorted based on levels of GFP using a FACSVantage/Diva sorter (BD Biosciences).

Expression of MIF in Mouse Motor Neurons and Analysis
To express MIF in motor neurons, a previously described protocol was followed with minor modifications (Kaec and Banker, 2006). Briefly, sorted GFP<sup>+</sup> motor neurons were plated at a density of 15,000 cells per well on a laminin-coated 96-well plate. Twelve hours after plating, the cells were infected with lentivirus to overexpress transgenes (40 viral particles per motor neuron). Motor neuron cultures were allowed to continue for another 5 days, with half of the media being replaced every other day. RFP could be detected after 72 hr postinfection. At various time points during the culture of motor neurons, images were recorded using a fully automated IN Cell 6000 cell imager (GE Healthcare) as previously reported (Meyer et al., 2014). Images were further processed with the Developer and Analyzer software package (GE Healthcare) for survival counts and neurite length measurements. Unless otherwise noted,
images shown represent 120 hr postinfection. All counts were performed in triplicate and repeated at least three times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.02.034.

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Supplemental Information

Macrophage Migration Inhibitory Factor as a Chaperone Inhibiting Accumulation of Misfolded SOD1

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Inventory of Supplemental Information for Israelson et al.

1. Supplemental Figures
   Figure 1S. A proteinase K resistant factor present in liver cytosol suppresses association of mutant SOD1 with mitochondria (associated to Figure 1).
   Figure 2S. MIF protein abundance in the spinal cord remains constant during mutant SOD1-mediated disease course (associated to Figure 2).
   Figure 3S. Dose dependent binding of mutant SOD1 to mitochondria (associated to Figure 3).
   Figure 4S. MIF suppresses accumulation of misfolded mutant SOD1 in human SH-SY5Y cells (associated to Figure 4).
   Figure 5S. Determination of the fraction of misfolded SOD1 initially present in recombinant mutant SOD1 (associated to Figure 3 and Figure 5).
   Figure 6S. MIF suppresses accumulation of newly formed misfolded mutant SOD1 (associated to Figure 5).
   Figure 7S. A low level of MIF in motor neuron cell bodies accompanied by accumulation and upregulation of MIF in astrocytes in early symptomatic SOD1\textsuperscript{G93A} rats (associated to Figure 6).

2. Supplemental Figure Legends

3. Supplemental Experimental Procedures
   Subcellular Fractionation
   Protein Purification
   Immunoprecipitation
   Microscale Thermophoresis (MST)
   DSE2 Antibodies
   Cell Culture and plasmids
   Immunoblotting
   Immunofluorescence
   Purification of cell type-specific mRNA from bacTRAP mice
Quantitative RT-PCR

4. Supplemental References
Supplementary Figure 1. A factor present in unaffected tissues suppresses the association of mutant SOD1 with mitochondria (associated to Figure 1).
Supplementary Figure 2. MIF protein abundance in the spinal cord remains constant during mutant SOD1-mediated disease course (associated to Figure 2).

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Supplementary Figure 3. Dose-dependent binding of mutant SOD1 with mitochondria (associated to Figure 3).

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Transfected with: hSOD1\(^{WT}\) or hMIF\(^{WT}\) + hSOD1\(^{G93A}\) or hSOD1\(^{G85R}\) SH-SY5Y cells → Clarified tissue homogenate → Immunoprecipitation with B8H10 misfolded SOD1 antibodies → SDS-PAGE → Immunoblot

Supplementary Figure 4. MIF suppresses accumulation of misfolded mutant SOD1 in human SH-SY5Y cells (associated to Figure 4).

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Supplementary Figure 5. Determination of the fraction of misfolded SOD1 initially present in recombinant mutant SOD1 (associated to Figure 3 and Figure 5).

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Supplementary Figure 6. MIF suppresses accumulation of newly formed misfolded mutant SOD1 (associated to Figure 5).

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Supplementary Figure 7. A low level of MIF in motor neuron cell bodies accompanied by accumulation and upregulation of MIF in astrocytes in early symptomatic SOD1<sup>G93A</sup> rats (associated to Figure 6).

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Supplementary Figure legends

Figure S1. A proteinase K resistant factor present in liver cytosol suppresses association of mutant SOD1 with mitochondria. This supplemental Figure is associated to Figure 1. (A) Schematic of protocol for testing the properties of liver cytosol in inhibiting mutant SOD1 association with mitochondria. (B) Spinal cord, brain or liver cytosol from SOD1\textsuperscript{G93A} expressing rats was incubated with non-transgenic liver derived mitochondria and the mitochondria subsequently recovered and analyzed by immunoblotting for acquisition of mutant SOD1. The liver cytosol fraction was subjected to different treatments (EGTA to chelate the calcium, proteinase K to digest most proteins, heat at 70°C, phenylethynesulfonamide to inhibit Hsp70 or 17-allylamino-demethoxygeldamycin to inhibit Hsp90) in order to characterize the activity. Immunoblotting for cytochrome c was used to verify numbers of mitochondria recovered from each assay. (Bottom) Input cytosols were immunoblotted to identify the initial levels of human mutant SOD1 and endogenous rat SOD1. Some lanes were not loaded in the same order as shown in the figure.

Figure S2: MIF protein abundance in the spinal cord remains constant during mutant SOD1-mediated disease course. This supplemental Figure is associated to Figure 2. Level of MIF protein accumulation was assessed before disease initiation or end stage disease in transgenic mice expressing mutant SOD1\textsuperscript{G85R}, SOD1\textsuperscript{G93A}, or SOD1\textsuperscript{G37R}, or in age matched non-transgenic control mice. MIF levels were detected on parallel immunoblots of whole spinal cord lysates after probing with distinct antibodies recognizing MIF.

Figure S3: Dose dependent binding of mutant SOD1 to mitochondria. This supplemental Figure is associated to Figure 3. (A) Schematic of a protocol to assess dose dependent binding of mutant SOD1 to mitochondria. (B) Immunoblotting of lysates of non-transgenic liver
mitochondria recovered from the protocol in (A) and assayed for dose-dependent association of recombinant SOD1<sup>G93A</sup>. Immunoblotting for VDAC1 was used to verify amount of mitochondria added/recovered.

**Figure S4. MIF suppresses accumulation of misfolded mutant SOD1 in human SH-SY5Y cells.** This supplemental Figure is associated to Figure 4. (A) Schematic of a protocol to test if expression of MIF can act to suppress accumulation of misfolded mutant SOD1 within SH-SY5Y cells. (B) MIF was expressed by transient transfection in human SH-SY5Y cells also transfected to express wild type or mutant human SOD1. Misfolded SOD1 was detected by immunoblotting of immunoprecipitates produced with the B8H10 antibody which recognizes epitopes within exon 3 that are buried in correctly folded SOD1. Expression of MIF and SOD1 wild type or mutant was determined by immunoblotting in the initial cytosol fractions.

**Figure S5. Determination of the fraction of misfolded SOD1 initially present in recombinant mutant SOD1.** This supplemental Figure is associated to Figure 3 and Figure 5. (A) Schematic of a protocol to determine the proportion of misfolded SOD1 present in the recombinant mutant SOD1<sup>G93A</sup>. (B) Misfolded SOD1 was determined by immunoblotting of consecutive dilutions of immunoprecipitates (generated with B8H10 antibody) of recombinant hSOD1<sup>G93A</sup>. Immunoblotting was also used to determine hSOD1<sup>G93A</sup> levels remaining in the unprecipitated (unbound) fraction. The percentage of misfolded SOD1 can be determined by matching the intensity of the immunoblot signal of the immunoprecipitate (marked B in lane 1) to the corresponding signals representing known percentages of the unbound SOD1 (lanes 2-6). Three independent repeats are shown.
**Figure S6. MIF suppresses accumulation of newly formed misfolded mutant SOD1.** This supplemental Figure is associated to Figure 5. (A) Schematic of a protocol to determine if purified recombinant MIF, Hsc70 and aB-crystallin can suppress the accumulation of newly formed misfolded SOD1 detected with B8H10 antibody. (B) Accumulation of misfolded SOD1 was determined by immunoblotting of immunoprecipitates of recombinant hSOD1\textsuperscript{G93A} performed with the B8H10 antibody. The unbound fraction was subjected to a second immunoprecipitation performed after incubation in the absence (lanes 1 and 3) or presence (lane 2) of recombinant MIF, (lane 4) aB-crystallin or (lane 5) Hsc70. Immunoblotting was also used to determine MIF, Hsc70 and aB-crystallin levels remaining in the unbound fraction of each immunoprecipitation assay.

**Figure S7. A low level of MIF in motor neuron cell bodies accompanied by accumulation and upregulation of MIF in astrocytes in early symptomatic SOD1\textsuperscript{G93A} rats.** This supplemental Figure is associated to Figure 6. (A) Localization of MIF in the ventral horn of the lumbar spinal cord of a non-transgenic rat using an antibody recognizing the N-terminus of MIF, along with identification of neurons (NeuN) and misfolded SOD1 (B8H10). (B) Immunostaining for MIF in the lumbar spinal cord of an early symptomatic SOD1\textsuperscript{G93A} rat one week after the first muscle fibrillation, along with simultaneous detection of neurons (NeuN) and misfolded SOD1. (C) Colocalization of MIF with astrocytes identified by GFAP antibody in the lumbar spinal cord of a non-transgenic rat, with simultaneous detection of misfolded SOD1 (B8H10). (D) Accumulation of MIF in astrocytes of the lumbar spinal cord of an early symptomatic SOD1\textsuperscript{G93A} rat. Higher magnification insets are included in the second row of each panel. Images are representative of immunostaining observed in n=5 non-transgenic rats and n>3 SOD1\textsuperscript{G93A} rats. Scale bar, 100 μm.
Supplemental Experimental Procedures

Subcellular Fractionation

Mitochondria were purified as previously described (Israelson et al., 2010; Vande Velde et al., 2008). Tissues were homogenized on ice in 5 volumes of ice-cold homogenization buffer (HB) composed of 210 mM mannitol, 70 mM sucrose, 1 mM EDTA-(Tris) and 10 mM Tris-HCl (pH 7.2). Homogenates were centrifuged at 1000 x g for 10 min. Supernatants were recovered, and pellets were washed with ½ volume HB and centrifuged at 1000 x g. Supernatants were pooled and centrifuged at 12,000 x g for 15 min to yield a crude mitochondrial pellet. The supernatant was used to make cytosolic fractions by further centrifugation at 100,000 x g for 1 hour. The mitochondria were gently resuspended in HB and then adjusted to 1.204 g/ml Optiprep (iodixanol) and loaded on the bottom of a polycarbonate tube. Mitochondria were overlaid with an equal volume of 1.175 g/ml and 1.079 g/ml Optiprep and centrifuged at 50,000 x g for 4 h (SW-55; Beckman). Mitochondria were collected at the 1.079/1.175 g/ml interface and washed once to remove the Optiprep. Optiprep stock solution was diluted in 250 mM sucrose, 120 mM Tris-HCl (pH 7.4), 6 mM EDTA plus protease inhibitors.

Liver was homogenized in 5 volumes of ice-cold homogenization buffer (HB) on ice. Homogenates were centrifuged at 1000 x g for 5 min. Supernatants were recovered, and centrifuged again at 1000 x g for 5 min. Supernatant was centrifuged at 12,000 x g for 10 min to yield a crude mitochondrial pellet. These mitochondria were resuspended in HB (without EDTA) and centrifuged again at 12,000 x g for 10 min. The pellet was resuspended in a small volume of HB without EDTA.
Protein Purification

Baculoviral stocks expressing human wild type and mutants for SOD1 were provided by Dr. Lawrence Hayward (University of Massachusetts). Recombinant hSOD1
\(^{\text{wt}}\), hSOD1
\(^{G93A}\) and hSOD1
\(^{G85R}\) were expressed in sf-9 cells and purified using a Hydrophobic Interaction Chromatography (HIC) and Ion Exchange Chromatography (IEX), as described previously (Hayward et al., 2002).

Immunoprecipitation

Purified recombinant SOD1 proteins (4 µg) or isolated cytosolic fraction (150 µg) were solubilized in immunoprecipitation (IP) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 plus protease inhibitors] and incubated overnight with B8H10 (MediMabs) or DSE2 antibodies previously crosslinked to Dynabeads protein G (Invitogen) with dimethyl pimelimidate (Pierce) according to the manufacturer’s instructions. The beads were magnetically isolated and washed three times with IP buffer. Samples were eluted with boiling in 2x sample buffer.

Microscale Thermophoresis (MST)

MST analysis was performed using a NanoTemper Monolith NT.115 apparatus, as recently described (Wienken et al., 2010; Zillner et al., 2012). Briefly, purified MIF was fluorescently labeled using the NanoTemper protein-labeling kit green (NanoTemper Technologies, Munich, Germany). A constant concentration of 200 nM of MIF was incubated for 20 min at 20 °C in the dark with different concentrations of mutant SOD1
\(^{G93A}\) (2.4 nM to 80 µM) in PBS containing 0.05% Tween 20. Afterward, 3–5 µl of the samples were loaded into a glass
capillary (Monolith NT Capillaries), and thermophoresis analysis was performed (light-emitting diode 20%, IR laser 20%) (Wienken et al., 2010; Zillner et al., 2012). $K_D$ were calculated using the mass action equation via the NanoTemper software from duplicate reads of triplicate experiments.

**DSE2 Antibodies**

DSE2 antibodies were kindly provided by Neil R. Cashman. Disease-specific epitopes (DSE) of SOD1 were as previously described (Israelson et al., 2010; Vande Velde et al., 2008). The epitope recognized by the antibodies were predicted to be exposed and unstructured during misfolding or metal depletion (Rakhit et al., 2007). Two independent IgG monoclonal clones (3H1 and 8D1) were selected by reactivity to the DSE2 peptide (comprising the electrostatic loop of hSOD1; residues 125-142), to denatured and/or oxidized hSOD1 *in vitro*.

**Cell culture and Plasmids**

To generate pCDNA-hMIF and pCDNA-hMIF$^{C60S}$ human macrophage migration inhibitory factor (MIF) cDNA (Jurgen Bernhagen, University Hospital RWTH Aachen, Germany), was amplified by PCR and inserted into pCDNA3.1(-) using BamH-I and Xba-I sites. pEGFP-hSOD1$^\text{wt}$, pEGFP-hSOD1$^{G93A}$ and pEGFP-hSOD1$^{G85R}$ were kindly provided by Jean Pierre Julien (Laval University, Canada). To generate mammalian expression plasmids carrying human SOD1 with an EGFP tag at the carboxyl terminus, SOD1 cDNA was fused to pEGFP-N2 (BD Bioscience, Palo Alto, CA, USA) at BamHI/EcoRI sites as previously described (Urushitani et al., 2008). Untagged versions of the above plasmids (pCI-hSOD1$^\text{WT}$, pCI-hSOD1$^{G93A}$ and pCI-hSOD1$^{G85R}$) were generated by inserting human SOD1 constructs into pCI-NEO (Promega) between EcoRI and NotI sites. NSC-34 cells were grown at 37°C and 5% CO$_2$ in DMEM.
supplemented with 10% tetracycline-free FBS and penicillin/streptomycin. SH-SY5Y cells were grown at 37°C and 5% CO₂ in DMEM (Biological Industries (B.I.)) supplemented with FBS (10%) (B.I.), L-Glutamine (2 mM) (B.I.) and penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (B.I.). Transfection was performed using Lipofectamine-2000 (Invitrogen) or TurboFect (Thermo) according to manufacture protocol. When co-transfections were performed, empty plasmids were always transfected as controls. The cells were collected 24 hours after transfection and analyzed for misfolded protein accumulation. For the mouse motor neuronal cultures, cells were infected with a CMV-hMIF-IRES-dsRED lentivirus, which was subcloned from the pcDNA3.1 construct using Pme1 sites.

**Immunoblotting**

Proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes and probed with various antibodies as indicated. Commercial antibodies include goat anti-SOD1 (C-17; SCB); sheep anti-SOD1 (Calbiochem), monoclonal anti-VDAC/porin 31HL (Calbiochem), goat anti-VDAC1 (N-18; SCB), goat anti-MIF (N-18, SCB), rabbit anti-MIF (FL-115, SCB), rabbit anti-cytochrome c (BD Biosciences), rabbit anti-CyPA (H-24; SCB), goat anti-CTH (P-15; SCB), monoclonal anti-ADH (A-8; SCB), goat anti-AAT (V-14; SCB), monoclonal anti-profilin-1 (C-2; SCB), rabbit anti-arginase (H-52; SCB), goat anti-Hsp27 (C-20; SCB), monoclonal anti-GPx (D-12; SCB), monoclonal Hsc70 (1B5; MBL), monoclonal anti-aB-crystallin (C-5; SCB). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG secondary antibodies (Jackson Immunochemicals) were used and detected by ECL (GE Biosciences).

In Supplementary Figure 2, whole tissue extracts were prepared from spinal cords by lysis in 1x TBS, 1% Triton X-100, 5mM EDTA (pH 7.4) with protease inhibitors, using a glass
homogenizer. Lysates were cleared of debris by centrifugation at 13,000 rpm for 10 minutes at 4°C, and then prepared for gel electrophoresis, loading 20ug per well in Criterion 4-12% Bis-Tris gradient gels from Bio-Rad. MIF was detected using an antibody from Invitrogen (36-7401 rabbit polyclonal antibody, 1:2000), or from Santa Cruz (N-18, goat polyclonal antibody, 1:300).

**Immunofluorescence**

Rats were anesthetized with pentobarbital and phenytoin and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in PBS. The spinal cords were dissected and post-fixed in 4% formaldehyde in PBS overnight at 4°C, and then cryoprotected in 30% sucrose in PBS. Free floating sections (40um-thick) were stained using standard protocols as described before (Sevc et al., 2013). Sections were immunostained overnight with antibodies made in PBS with 0.2% Triton-X100: goat anti-choline acetyltransferase (ChAT, Chemicon), mouse anti-neuronal nuclei antigen (NeuN, Chemicon), anti-glial fibrillary acidic protein (GFAP), mouse anti-misfolded SOD1 (B8H10, Medimabs), or anti-MIF (36-7401 rabbit polyclonal antibody from Invitrogen, or N18 goat polyclonal antibody from Santa Cruz Biotechnology). Following overnight incubation in primary antibodies, sections were washed three times in PBS and incubated with fluorescent conjugated secondary donkey anti-mouse, donkey anti-rabbit, or donkey anti-goat antibodies, and DAPI for nuclear staining. Images were acquired on an Olympus FluoView FV1000-MPE confocal microscope, using a 60x oil objective. Z-stack images were obtained from 10-12 optical sections with 0.4μm spacing, and were reconstructed in ImageJ2. Scanning settings for individual channels were kept constant across the samples and final image properties (intensity, brightness, contrast) were adjusted using the same settings for all images.
Purification of cell type-specific mRNA from bacTRAP mice

Dissected mouse spinal cord was immediately homogenized in ice-cold polysome extraction buffer (20 mM HEPES [pH7.4], 150 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 100 μg/ml cycloheximide, protease inhibitors, and RNase inhibitors). Homogenates were centrifuged for 10 min at 2000 × g at 4°C, and NP-40 and 1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine (DHPC, Avanti Polar Lipids) were added to the supernatant at a final concentration of 1% and 30 mM. The lysates were centrifuged for 15 min at 13000 × g at 4°C after incubation on ice for 5 min. Two monoclonal GFP antibodies (Htz-GFP19C8, Htz-GFP19F7, MSKCC MACF) coated protein G Dynal magnetic beads (Invitrogen) were added to the supernatant, and incubated at 4°C with rotation overnight. Beads were subsequently washed five times with high-salt polysome wash buffer (20 mM HEPES [pH7.4], 350 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 1% NP-40 and 100 μg/ml cycloheximide). The RNA bound on the beads were extracted by Absolutely RNA Nanoprep kit (Stratagene) and quantified by RiboGreen RNA assay (Invitrogen).

Quantitative RT-PCR

For first-strand cDNA synthesis, random hexamers were used with High-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR reactions were performed with 3-4 biological replicates for each group and two technical replicates using the iQ SYBR green supermix (Bio-Rad) on the iQ5 multicolor real-time PCR detection system (Bio-Rad). The data were analyzed using the iQ5 optical system software (Bio-Rad; version 2.1). Expression values were normalized to the control gene Dnaja2. Relative expression values were normalized to that in motor neurons. Inter-group differences were assessed by two-tailed Student's t-test.
Supplementary References


