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From Charcot to SOD1: Mechanisms of Selective Motor Neuron Death in ALS

Review

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The sixth anniversary has just passed since the landmark discovery (Rosen et al., 1993) that mutation in the enzyme superoxide dismutase 1 (SOD1) is a primary cause of a proportion of a dominantly inherited form of amyotrophic lateral sclerosis (ALS), more familiarly known in the United States as Lou Gehrig's disease. First described by Charcot 124 years before that, the primary characteristic of this disease is the selective degeneration and death of upper and lower motor neurons, initiating in mid adult life and almost invariably progressing to paralysis and death over a 1–5 year time course. While dominant inheritance accounts for only about 10% of the instances of disease, and SOD1 mutations comprise fewer than 20% of those (reviewed by Brown, 1995), the genetics provided an enormous injection of hope for dissecting the mechanism of disease, its neuronal selectivity, and the timing of disease onset and for identifying therapeutic approaches.

Four primary hypotheses for contributors to disease mechanism have now emerged: (1) oxidative damage, an idea obviously provoked by the discovery of SOD1 mutations; (2) axonal strangulation from neurofilamentous disorganization, supported by misaccumulated neurofilaments as a hallmark of pathology in many cases of sporadic and SOD1-mediated familial disease; (3) toxicity arising from intracellular aggregates and/or failure of protein folding or degradation, a common feature of SOD1 mutant–mediated disease; and (4) excitotoxicity from aberrant handling of glutamate, particularly arising from missplicing of a glutamate transporter mRNA. Where do these issues now lie?

SOD1 Mutants Confer a Toxic Property(ies)

That mutation in SOD1 could be a primary cause of selective motor neuron degeneration was initially anything but obvious. Ubiquitously expressed, the known activity of this 153-amino-acid, cytoplasmic homodimer is to convert superoxide, produced primarily by errors of oxidative phosphorylation in mitochondria, to water and hydrogen peroxide. Catalysis is mediated in two asymmetric steps by an essential copper atom, which is alternately reduced and then oxidized by superoxide (Figure 1A). While early measurements of SOD1 activity in patient blood suggested loss of enzymatic activity to be central to the mechanism of disease, an initial transgenic mouse expressing familial ALS–linked mutant $SOD1^{G93A}$ (i.e., glycine substituted to alanine at position 93) developed progressive motor neuron disease despite markedly elevated SOD1 activity levels (Gurney et al., 1994). This has been extended by three additional sets of mice expressing different SOD1 mutants, which in each case provoke disease with elevated (Wong et al., 1995) or unchanged (Ripps et al., 1995; Bruijn et al., 1997b) SOD1 activity, while SOD1 null mice live to adulthood and do not develop motor neuron disease (Reaume et al., 1996). Further, some mutants such as $SOD1^{G37R}$ retain full specific activity (Borchelt et al., 1994), while neither the age of onset nor rapidity of progression of human disease correlate with dismutase activity levels (Cudkowicz et al., 1997). The inescapable conclusion from this abundance of evidence is that the mutants have acquired one or more toxic properties, irrespective of the amount of SOD1 activity each retains.

The Copper-Mediated, Oxidative Damage Hypothesis

Of the proposals to explain how more than 65 different mutations scattered throughout the SOD1 polypeptide can mediate the same neurodegenerative disease, the most prominent one ascribes toxicity to less tightly folded enzymes that perform aberrant copper-mediated chemistry due to greater access of abnormal substrates to the active site copper. A slight modification is that the mutants may handle the copper clumsily, frequently releasing it, with the free copper catalyzing unwanted oxidative chemistry. Consistent with aberrant copper-mediated catalysis, a common property of the mutants, including those in histidines that directly coordinate the copper, is that they do bind the copper in at least one in vivo setting (Corson et al., 1998).

A first aberrant substrate proposed (Beckman et al., 1993) was peroxynitrite ($^{-}$ONOO), which can form spontaneously from superoxide and nitric oxide and, when used as a substrate, yields tyrosine nitration (Figure 1B). Despite divergent evidence from several groups (primarily using immunocytochemistry) for whether elevated levels of the predicted nitrotyrosine are present in either SOD1-mediated disease in mouse and humans, evidence from manipulating SOD1 activity levels in mice makes the peroxynitrite hypothesis unlikely to play a central role. In mice that develop disease from expressing a mutant ($SOD1^{G85R}$) that confers only a ~10% increase in SOD1 activity over the endogenous, wild-type SOD1, neither elimination nor elevation (by ~6-fold) of wild-type SOD1 affected disease onset, progression, or pathology (Bruijn et al., 1998). The insensitivity of toxicity to SOD1 activity levels is inconsistent with damage arising from superoxide or any spontaneous reaction product of it (such as $^{-}$ONOO), since absence of the wild-type protein would accelerate toxicity by raising superoxide levels and/or forcing all catalysis through the mutant, while elevating it would do the opposite. This was clearly not the case, at least for the $SOD1^{G85R}$ mutant.

A second proposed aberrant substrate is hydrogen peroxide, the normal end product released from the oxidized form of the enzyme (Cu^{2+}–SOD1). Use of peroxide by the reduced Cu^{+}–SOD1 form, on the other hand, may produce the extraordinarily reactive hydroxyl radical (Figure 1C), thereby leading to a cascade of peroxidation. A 2- to 4-fold increased utilization in vitro of

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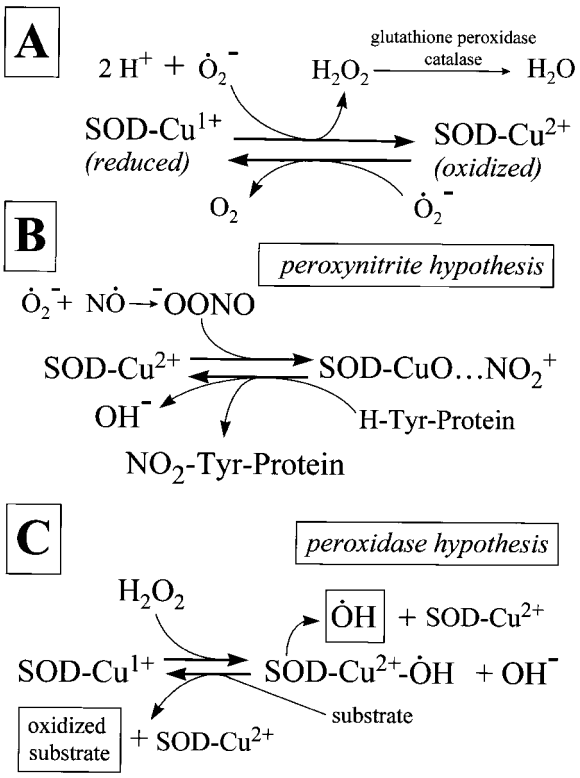

Figure 1. SOD1 Chemistries

(A) Normal SOD1 chemistry: SOD1-mediated dismutation of superoxide in two asymmetric steps.

(B and C) Proposed toxic chemistries from use of aberrant substrates.

(B) Protein nitration from $^{-}$ONOO.

(C) Hydroxyl radical formation from hydrogen peroxide.

hydrogen peroxide as a substrate by two mutants (relative to wild-type SOD1) has been reported (Wiedau-Pazos et al., 1996), although this has been directly challenged on technical grounds (Singh et al., 1998). Also, whether a higher level of peroxidation will arise from such a mechanism is also not settled, since the fewfold increase by the mutant SOD1 (compared to wild type) in peroxide usage could easily be more than counterbalanced by the decreased stability (and hence accumulated level) of many mutants.

Testing the Oxidative Hypothesis

In testing the proposals for aberrant substrates, increased levels of markers of oxidative damage (protein carbonyls, products of hydroxyl radical damage, protein nitration) have been reported in disease in some (Ferrante et al., 1997; Andrus et al., 1998) of the transgenic mice but not others (Bruijn et al., 1997a), and in some but not all examples of sporadic or SOD1-mediated familial ALS (E.g., Bowling et al., 1993). Amid such divergent findings, no conclusion can yet be drawn as to whether, or to what degree, oxidative damage plays a role in disease. And there may, of course, be yet unidentified alternative substrates of mutant SOD1 that initiate a cascade of oxidatively damaged products that have been overlooked by the conventional assays.

Whatever the case, the seminal discovery that copper acquisition by SOD1 in yeast requires CCS, a specific copper chaperone for SOD1 (Culotta et al., 1997), has allowed design of a test in a single experiment of all possible, copper-mediated toxicities. Since both the human wild-type and mutant SOD1s apparently load copper in vivo through the action of a mammalian CCS (Corson et al., 1998), production of SOD1 mutant–expressing mice that are also deleted for the single mammalian CCS will directly test the requirement for copper (and any catalyzed oxidative damage) in SOD1 mutant toxicity. This experiment, which has the power to disprove copper-mediated oxidative mechanisms should toxicity prove insensitive to copper loading, is well underway in at least one group (P. Wong, personal communication), with a clear answer anticipated by early next year.

Neurofilaments as Contributors and Risk Factors in ALS

From early reports of pathology of both sporadic (Hirano et al., 1984a) and familial (Hirano et al., 1984b) ALS, neurofilament misaccumulations in the cell bodies and proximal axons of motor neurons have been established to be hallmarks of the disease. With the further demonstration that transgenes encoding mutant neurofilament subunits could directly cause selective degeneration and death of motor neurons and the ensuing axonal disorganization, this led to the proposal that damage to neurofilaments was directly involved in ALS pathogenesis.

A pair of genetic experiments has now demonstrated that this is true even in the case of disease initially provoked by mutant SOD1. By using gene disruption to eliminate neurofilaments from mice expressing the $SOD1^{G85R}$ mutant, onset and progression of disease has been found to be slowed by 40 days, despite the significant initial disadvantage of loss of ~15% of motor neurons early in postnatal life arising from absence of neurofilaments (Williamson et al., 1998). Even more provocative is that decreasing the axonal amount of neurofilaments, by trapping most in the cell bodies through transgene-increased expression of the large neurofilament subunit NF-H, extends life span in $SOD1^{G37R}$ mice by 6 months (a 65% increase in longevity) (Couillard-Despres et al., 1998). While several other strategies have successfully slowed disease in SOD1 mice (see below), this last neurofilament strategy represents by far the most striking slowing of disease. Indeed, since neurofilaments are determinants of axonal diameter and only the largest caliber, neurofilament-rich motor axons are lost in human disease (Kawamura et al., 1981) or SOD1 mutant–mediated disease in mice (Bruijn et al., 1997), the simple view is that neurofilament content represents one of the factors underlying selective vulnerability.

Genetic Evidence for Neurofilament Mutations as Cause or Risk Factor in ALS

The evidence that in mice mutant neurofilaments could directly provoke motor neuron disease and the accompanying neurofilamentous misaccumulation fueled efforts to test whether neurofilament mutations underlie disease. Early efforts focused on the 80% of familial

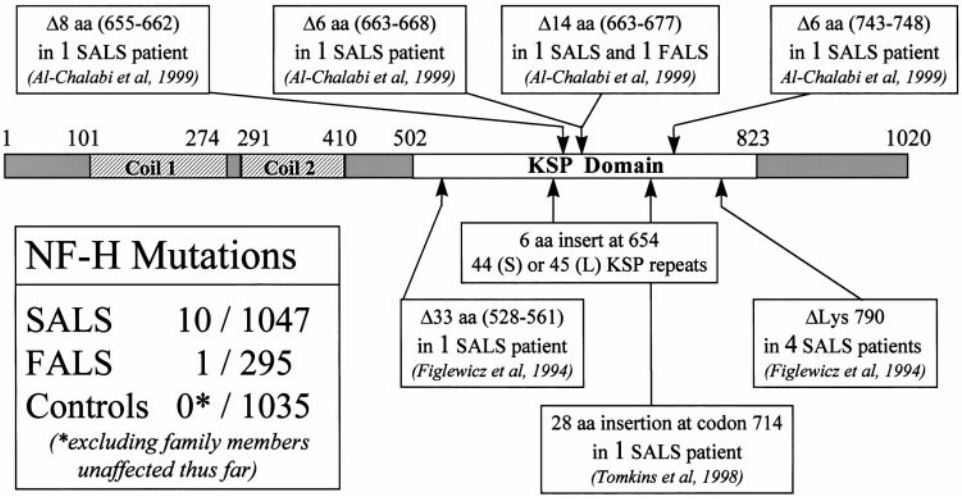

Figure 2. Mutations in Sporadic and Familial ALS Found in the Gene Encoding the Large Neurofilament Subunit NF-H

ALS not arising from SOD1 mutation but did not identify neurofilament mutants. Subsequent efforts examining the repetitive tail domain of the large neurofilament subunit NF-H, especially the extensive study of Al-Chalabi et al. (1998), have now identified a set of small in-frame deletions or insertions in ~1% of more than 1300 ALS patients, almost all of which appear in "sporadic" cases (Figure 2). Excluding family members not yet affected, no similar mutants have been seen in a comparable number of control DNAs. Search of this one domain alone has thus yielded mutations in the overall patient population about half as frequent as SOD1 mutants. It should be realized that variation in timing of disease onset and incomplete penetrance can befuddle identifying an underlying genetic component. While the known neurofilament sequence variants are surely not by themselves capable of provoking disease with high penetrance, the collective evidence now strongly hints that variants in neurofilaments are, at the least, important risk factors for apparently sporadic disease.

Axonal Strangulation from Chronic Deficits in Transport

Because biosynthesis of macromolecules is almost completely restricted to neuronal cell bodies, axonal transport is especially crucial to the long, large caliber motor axons. The known neurofilament-dependent slowing of axonal transport, combined with the prominent misaccumulation of neurofilaments in ALS, suggested that an important aspect of toxicity may arise from damage to transport. Chronic deficits in transport of selective cargoes of slow transport have now been seen as very early features, initiating up to 5 months prior to clinical disease onset in mice expressing $SOD1^{G85R}$ and $SOD1^{G37R}$ mutants (Williamson and Cleveland, 1999). Deficits in fast transport have been seen prior to disease onset in $SOD1^{G93A}$ mice (Warita et al., 1999). Thus, whether arising from direct damage to the machinery or cargoes of transport or secondary to other primary damage, a common deficit from SOD1 mutants that provoke different pathologies is chronically compromised delivery of selected transport cargoes. Since neurofilaments are a major cargo whose presence slows the speed of slow transport, this offers at least a partial explanation for the vulnerability of the large motor axons at risk. The motor neurons, with large, neurofilament-rich axons that in humans are up to a meter in length, are among the body's longest and largest cells (up to 5000 times the volume of a typical cell), almost all components of which must be transported into and along the axon.

Intracellular Aggregates of SOD1: Are They Toxic?

Studies of several neurodegenerative diseases (Alzheimer's disease, prion diseases, triplet repeat expansion diseases such as Huntington's) have revealed a common feature: protein aggregates. This commonality has fueled a long-standing debate over whether these aggregates are key to the pathogenesis, harmless byproducts, or potentially beneficial through sequestration of aberrant products. The likelihood that aggregates might be contributors to ALS was significantly boosted by the finding that all examples of SOD1 mutant–mediated disease in mice develop prominent, cytoplasmic, intracellular inclusions in motor neurons, and in some cases within the astrocytes surrounding them as well. These aggregates develop by the onset of clinical disease, in some cases (e.g., $SOD1^{G85R}$ mice) representing the first pathologic sign of disease, and later increase markedly in abundance during disease progression (Bruijn et al., 1997).

As to their composition, the striking feature is that aggregates are intensely immunoreactive with antibodies to SOD1, a feature common not only to all instances of disease in mice but also to several SOD1-mediated human examples (see Bruijn et al., 1998, and references therein). Reported as well in a handful of sporadic cases (but not in others), such aggregates may prove to be a frequent feature even of sporadic disease, but a conclusive outcome awaits a more systematic test.

The aggregates are of two kinds. The first class contains those identifiable by conventional histological stains,

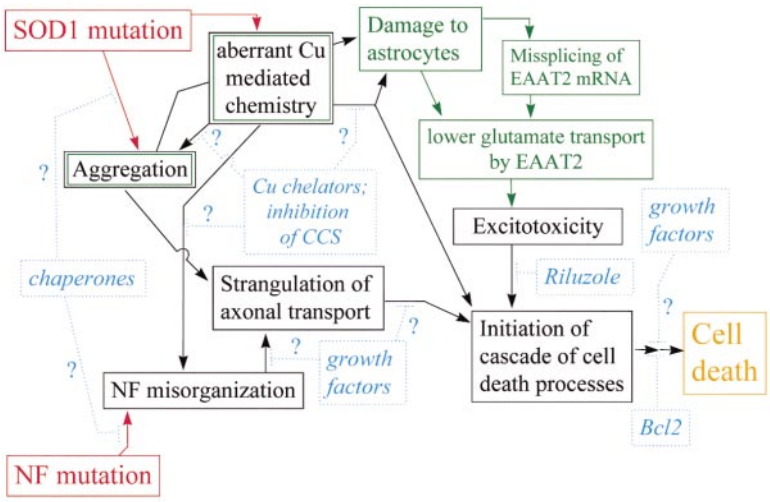

Figure 3. Proposed Mechanisms of, and Possible Therapies for, Motor Neuron Toxicity in ALS

Events within motor neurons are marked in black; events within astroctyes are in green. Potential therapeutic steps are marked in blue.

which most frequently reveal intense, concentrated SOD1 immunoreactivity throughout the inclusion or, less frequently, only at the periphery of it. The second class includes more diffusely staining perikaryal deposits. Mating SOD1 mutant mice to mice deleted at the endogenous SOD1 alleles or to mice expressing wild-type SOD1 to six times the normal level has shown that the timing, abundance, and staining intensities of the aggregates are independent of wild-type SOD1. While immunocytochemistry is among the world's worst quantitative tools, it seems safe to conclude that mutant SOD1 is one primary component of these aggregates.

Aggregation, or at least misfolded subunits, is thus apparently a characteristic of the SOD1 mutants. This has been reproduced selectively in motor neurons by microinjection of genes encoding mutant but not wild type SOD1. This approach has yielded what may be the best in vitro model of disease, including aggregates selectively in motor (but not sensory or hippocampal) neurons (Durham et al., 1997), followed by cell death that can be slowed by apoptosis inhibitors. Aspects of toxicity may arise either through aberrant chemistry mediated by the misfolded aggregated mutants or through loss by coprecipitation of an essential component or components, for example, by saturating the protein degradation machinery and/or protein folding chaperones. Consistent with the latter, increasing the level of the stress-inducible chaperone HSP 70 reduced mutant-driven aggregates and ameliorated toxicity in this in vitro cell model (Bruening et al., 1999). It should be noted that the outcome of the test of influence of copper loading on toxicity (see above) will probably bear directly on distinguishing the potential contributions of copper catalysis versus aggregation.

Excitotoxicity and Glutamate Transporters: Are Astrocytes, Not Neurons, to Blame?

Glutamate-mediated excitotoxicity due to repetitive firing and/or elevation of intracellular calcium by calcium-permeable glutamate receptors has been frequently implicated in neuronal death. High-affinity glutamate transport is the primary means of rapid recovery of synaptic glutamate, thereby suppressing this toxicity. For spinal motor neurons, glutamate recovery is the job of astrocytes, which synthesize the major spinal cord transporter EAAT2 (previously known as GLT-1). For ALS, early evidence for involvement of glutamate came from the demonstration of selective, local loss of EAAT2: affected tissue regions from about two-thirds of sporadic ALS patients were found to have up to 95% losses in EAAT2 levels and activity (Rothstein et al., 1995). Mechanistically, this does not arise from mutation but apparently from selective errors in splicing of the EAAT2 mRNA. This has been reported within affected, but not unaffected, regions of the two-thirds of sporadic patients that show diminished glutamate transport activity (Lin et al., 1998). In fact, evidence for many aberrantly spliced RNA species was reported, with two prominent products either with exon 9 skipped or intron 7 retained. Both make nonfunctional truncated products, and the latter dominantly inhibits normal EAAT2-mediated transport.

These powerful findings strongly support a role for astrocytic dysfunction as an important component of motor neuron death. Moreover, using DNA microinjection into *Xenopus* oocytes, ALS-linked SOD1 mutants, but not wild-type SOD1, were found to catalyze inactivation of EAAT2 in the presence of high levels of hydrogen peroxide (Trotti et al., 1999). When combined with prominent SOD1-immunoreactive aggregates in astrocytes in sporadic and SOD1-mediated disease in humans and mice, this promotes the satisfying hypothesis that damage within astrocytes initiated either by SOD1 mutation per se or by other (yet unidentified) initiators yields diminished defense from synaptic glutamate as a key aspect of the cascade of toxicity. Not only would this, at last, offer a direct mechanistic link between sporadic and SOD1-mediated disease, but it raises the possibility that the underlying toxicity could be partly or exclusively of astrocytic origin. This should now be clearly testable with transgenes expressed specifically in astrocytes or motor neurons.

But a crucial fly in the ointment is that the strikingly abundant EAAT2 mRNA missplicing has not yet been confirmed as a frequent feature of disease. Rather, one report (Nagai et al., 1998) finds similar levels of exon 8

skipping and intron 7 retention in normal as well as patient samples; so too have other reports (not yet in print) at recent neurology meetings. With the potential to directly link disease mechanism in the few SOD1-mediated examples with the majority of sporadic disease, confirming or refuting the effects on EAAT2 RNA metabolism should now be an immediate goal.

Treating Disease in Mice: Bcl-2 Expression, Caspase Inhibition, or Creatine Slow Disease

The Bcl-2 family of genes has been widely implicated in regulating cell death. Using transgenes to force chronically elevated postnatal expression of the cell death inhibitor Bcl-2, Przdeborski and colleagues demonstrated a significant (30–35 day) slowing of disease onset and extension of life span in SOD1^{G93A} mice, without effect on length of disease progression (Kostic et al., 1997). Mechanistically, it is not yet clear whether the positive effects of Bcl-2 are from acting as an antioxidant or as an inhibitor of apoptosis. Similar expression of a dominant, inhibitory caspase-1 (or ICE, the interleukin-1β-converting enzyme) yielded a more modestly extended life span (about 2 weeks) in the same SOD1^{G93A} mice (Friedlander et al., 1997).

Among other approaches to therapy (Gurney et al., 1996), administration of riluzole, which inhibits synaptic glutamate release, extended survival a very modest 10–15 days in SOD1^{G93A} mice. This is consistent with the correspondingly minimal benefit of riluzole in human therapy (Lacomblez et al., 1996). Little optimism arose from increased dietary vitamin E, an antioxidant defense against lipid peroxidation: this yielded no extension in life span in SOD1^{G93A} mice, although this may simply reflect that serum levels of vitamin E were not significantly elevated.

The best pharmacological intervention so far (Klivenyi et al., 1999) is the simple addition of creatine to the drinking water of the same SOD1^{G93A} mice. Long used by athletes hoping to enhance energy reserves in muscle, creatine yielded a dose-dependent extension in survival peaking at just under 4 weeks. How creatine provides this benefit mechanistically is by no means settled, but its availability at the local health food store means a safe bet is that it is it is already being taken widely.

A Cascade of Toxicity and Prospects for the Future

The overall pathways now known or suggested for disease mechanism and potential therapies are summarized in Figure 3. A toxic property of mutant SOD1 provokes selective neurotoxicity, probably through mutant-dependent aberrant copper-mediated chemistry and/or protein aggregation. Damage is found in both neurons (denoted in black) and astrocytes (denoted in green), but the degree to which disease is provoked from toxicity directly within either cell type is not settled. Indeed, whether toxicity is cell autonomous—that is, whether it must arise within each affected cell or whether it can be spread from mutant-expressing cells to normal ones, an especially important feature for sporadic disease—remains to be tested. Intracytoplasmic aggregates, comprised at least in part of mutant SOD1, are common features of disease and may reflect primary aspects of toxicity, either through strangulation of axonal transport or direct damage to astrocytes, perhaps provoking errors of mRNA maturation and glutamate metabolism. Neurofilament mutations can be direct causes of motor neuron disease in mice, and their misorganization by mutation (or other covalent damage) may also force chronic deficits in axonal transport that provoke initiation of a further cascade of cell death events. Potential interventions (denoted in blue in Figure 3) can be imagined at several levels. If copper chemistry is involved, attractive therapies could be inhibitors of the CCS required for loading copper onto SOD1. Alternatives would include a search for agents that affect neurofilament synthesis or assembly or anti-apoptotic agents to slow the cascade of events. And, we should not forget the simple, practical relief that creatine has provided in the SOD1^{G93A} mouse. Replication of these findings in other mutants and an expeditious human trial should be first priorities.

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