

## Forum Original Research Communication

# Oligomerization of Mutant SOD1 in Mitochondria of Motoneuronal Cells Drives Mitochondrial Damage and Cell Toxicity

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### Abstract

Increasing evidence indicates that the accumulation and aggregation of mutant Cu,Zn superoxide dismutase (mutSOD1) in spinal cord mitochondria is implicated in the pathogenesis of familial amyotrophic lateral sclerosis (FALS). Although the mechanisms underlying this effect are only partially understood, a deficit in the import mechanism of mutSOD1 and/or in its folding and maturation inside mitochondria is likely involved. To investigate this issue, we overexpressed mitochondria-targeted wild-type and mutSOD1s in neuronal cell lines. Mitochondria-targeted G93A mutSOD1 induces a significant impairment of mitochondrial morphology and metabolism, resulting in caspase-3 activation and cell death. These effects are paralleled by the formation of disulfide-linked, insoluble oligomers of mutSOD1 inside mitochondria. Overexpression of the copper chaperone for SOD1 (CCS) improves the solubility of cytosolic mutSOD1s, but has no effect or even worsens the insolubility of mitochondria-targeted G93A mutSOD1, indicating that CCS may increase the availability of an aggregating form of mutSOD1. Interestingly, prevention of the formation of such aggregates by removal of disulfide-bonded cysteines counteracts the effects produced by mutSOD1 accumulated inside mitochondria. Overall, our results demonstrate for the first time that aggregation of mutSOD1s into mitochondria is important for mutSOD1 to induce damage, although other forms of misfolded SOD1s might be involved. *Antioxid. Redox Signal.* 11, 1547–1558.

### Introduction

**A**MYOTROPHIC LATERAL SCLEROSIS (ALS) is a lethal disease which is sporadic in ~90% of cases (SALS), while the remaining 10% are associated to familial forms (FALS). In particular, >100 different mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) have been causally linked to FALS. A gained toxic function is probably shared by all the mutants (mutSOD1s), which are able to produce an ALS-like disease when introduced in the genome of rodents, yet the nature of this toxic property is still unknown (6). Abnormal aggregation of the mutant protein is an obvious candidate, since mutSOD1-containing aggregates have been observed in cellular and animal models of the disease (3, 10,

34), and extensive disulfide crosslinking between cysteine residues that are present in SOD1 may have a pivotal role in this phenomenon (2, 5, 19, 23). Although evidence has been provided showing that the removal of such aggregates by the elimination of reactive cysteines from mutSOD1s is able to rescue the viability of transfected cells (5, 23), transgenic mice that overexpress both the G93A mutSOD1 and its copper chaperone (CCS) die from an ALS-like disease at a time when cytosolic aggregates are not present, thus questioning about a causal role of such aggregates in the pathogenesis of the disease (28). Interestingly, these mice have a clearly enhanced mitochondrial damage (28, 29), and mitochondrial abnormalities appear to be shared by models and patients suffering from ALS (reviewed in Ref. 17). Compelling evidence has

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accumulated that uncontrolled accumulation of mutSOD1 inside the mitochondria of cells may be directly responsible for mitochondrial impairment (20, 22, 31, 32). For these reasons, the mechanism controlling the uptake of SOD1 in cell mitochondria has attracted much investigation. According to a model originally proposed by the group of V. Culotta, SOD1 can efficiently enter mitochondria only when it is *apo*- for both copper and zinc and reduced in the conserved disulfide (12). The retention of SOD1 within mitochondria is provided by the conversion of the immature polypeptide into an active *holo*-enzyme through the intervention of CCS, that is highly expressed in the intermembrane space (IMS) of mitochondria of yeast and mammalian cells (12, 21, 24). Kawamata *et al.* have recently provided strong evidence that this system acts also in mammalian cells, where the subcellular distribution of CCS dictates the localization of SOD1 (21). Moreover, the IMS of mitochondria has been recently shown to contain a machinery that efficiently traps incoming precursors through the introduction of disulfide bonds into newly imported precursor proteins, thereby locking them in a folded conformation (16). Experimental support for a role of this machinery in the oxidative folding and retention of SOD1 inside mitochondria is now available (21), thus pointing to cysteine residues of SOD1 as key mediators in the process of both import and folding inside mitochondria. Interestingly, disulfide-linked mutSOD1 oligomers have been described in the mitochondrial fraction of both spinal cord of ALS transgenic mice and cultured motor neuronal-derived cells (8, 10), suggesting that uncontrolled cysteine reactivity may underlie mutSOD1 protein oligomerization, aggregation and accumulation inside mitochondria.

Using mitochondria-targeted wild-type and G93A mutSOD1, we show here that in cultured cells of neuronal origin the accumulation of mutSOD1 inside mitochondria is sufficient to elicit mitochondrial damage and cell death, that this effect is strictly correlated to the formation of detergent-insoluble, disulfide-linked oligomers, and that the elimination of such oligomers by the removal of reactive cysteines strongly, but not completely, rescues the metabolism of mitochondria and the viability of cells. These results point to the accumulation of misfolded mutSOD1 inside cell mitochondria as a primary determinant of mutSOD1 motoneuronal toxicity in FALS.

## Materials and Methods

### Plasmid construction

The tetracycline-responsive vectors (pTRE2) coding for wild-type or mutSOD1s were described elsewhere (10). Wild-type and G93A SOD1, fused with the mitochondria-targeting signal of cytochrome c-1 (10), were cloned in pTRE2 vector with standard techniques and were used as templates for PCR site-directed mutagenesis (5) for the production of mitoG93A/C111S, mitoC111S, and mitoG93A/C111S/C6S mutants. Mouse CCS (accession number: NM 016892) cloned in a pCMV-Sport6 plasmid was purchased from RPDZ (Berlin, Germany).

### Cell culture, transfection, and transgenic mice

Mouse motoneuronal cell line NSC-34, stably transfected with the pTetON plasmid (Clontech, Palo Alto, CA) coding for the reverse tetracycline controlled transactivator, were

described elsewhere (10). The line designed NSC34/pTetON7, which displays a very low level of basal expression and high inducibility, was used to derive inducible cell lines expressing the human wild-type, G93A, mito-wtSOD1, mito-G93A, and mito-G93A/C111S SOD1 forms and cell lines constitutively expressing the mouse isoform of CCS, as previously described (10). NSC34-derived cell lines were grown in Dulbecco's modified Eagle's/F12 medium supplemented with 10% fetal calf serum tetracycline free (FCS Tet, Lonza, Basel, Switzerland), at 37°C in a atmosphere of 5% of CO<sub>2</sub> in air.

Fibroblasts from CCS +/+ and CCS -/- mice (a kind gift of J.D. Gitlin, The Johns Hopkins University School of Medicine, Baltimore, MD) and human neuroblastoma SH-SY5Y cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (EuroClone, Sizio, Italy). Transient expression of pTRE2-SOD1s vectors in NSC-34/pTetON7 was obtained by transfection with Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction, while in fibroblast and neuroblastoma cell lines the pTRE2-SOD1s were co-transfected with the pTetON plasmid. After a 3 h incubation in OptiMem (Invitrogen) with transfection reagents, cells were shifted in normal growth medium. Induction of SOD1 was obtained by adding to culture medium 1 µg/ml doxycycline for the indicated periods of time.

Transgenic mice expressing the human SOD1 gene with a G93A mutation (strain B6.Cg-Tg(SOD1-G93A)1Gur/J (15) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal studies were conducted in accordance with standard ethical guidelines. At a pre-symptomatic age (30 days) and at the end stage of the disease (150 days), transgenic mice were anesthetized and sacrificed, and spinal cords were dissected for the experiments.

### SOD1 solubility and aggregation assay

5–7 × 10<sup>5</sup> cells were scraped off the plate in culture medium, collected by centrifugation, washed with PBS, and resuspended in 70 µl of buffer A (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl, and protease inhibitor mixture, Sigma-Aldrich, St. Louis, MO) containing 0.5% Nonidet- P40. After 10 min of ice incubation, the lysates were centrifuged at 20,000 g for 10 min, and the supernatants were collected as detergent soluble fractions, whereas the pellet (insoluble fractions) was washed in PBS and resuspended in 70 µl of Laemmli sample buffer (62 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.05% bromophenol blue).

For the analysis of SOD1 oligomers, 100 mM iodoacetamide (Sigma-Aldrich) was added to the lysis buffer, to avoid uncontrolled oxidation of cysteine residues. The insoluble fractions were resuspended in buffer A containing 2% SDS and 100 mM iodoacetamide, heated to 100°C for 5 min, then centrifuged at 20,000 g. The supernatants were collected as the insoluble fractions. Protein content was determined using Bradford protein assay (Bio-Rad, Hercules, CA). Solubility assay and analysis of SOD1 oligomers in mitochondrial fraction from mouse spinal cord were performed through the same experimental procedures.

### Subcellular fractionation and mitochondria purification

Mitochondria were isolated from NSC-34-derived cell lines as previously reported (10). Mice spinal cord were

resuspended in mitochondrial buffer (MB, 200 mM mannitol, 50 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4) in a ratio 100 mg/ml, and homogenized in a potter homogenizer with a Teflon piston. Isolation of mitochondria was obtained accordingly to a previously reported method (13).

#### *Electrophoresis and Western blot analysis*

Standard SDS-PAGE was performed as described (10). Western blot was performed onto nitrocellulose membranes (Amersham Pharmacia Biosciences, Piscataway, NJ).

For the analysis of SOD1 oligomers, fractions were resuspended in Laemmli sample buffer without  $\beta$ -mercaptoethanol. Equal amounts of proteins were loaded onto 4–12% gradient Bis-Tris gels (NuPAGE, Invitrogen). After electrophoresis, the gels were soaked for 30 min in SDS-PAGE running buffer containing 5 mM Tris(2-carboxyethyl)phosphine (Invitrogen) and blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), as described by others (14).

After incubation in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 and 5% nonfat milk, filters were incubated for 2 h at room temperature with the indicated antibodies diluted in 2% nonfat milk, 0.1% Tween 20/TBS solution. Immunoreactive SOD1 was detected with a rabbit polyclonal anti-SOD1 antibody (1:5,000; Stressgen, San Diego, CA), which detects both human and mouse SOD1. The mouse CCS isoform was detected with a rabbit polyclonal anti-CCS antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). For the analysis of voltage-dependent anion channel (VDAC) expression level, the mouse monoclonal antibody anti-voltage-VDAC (1:5,000; Calbiochem, Darmstadt, Germany) was used, while the serine/threonine kinase AKT was detected using a mouse monoclonal antibody (1:500; Santa Cruz) and manganese superoxide dismutase (SOD2) with a rabbit polyclonal antibody (1:5,000; Stressgen).

Following extensive washing in 0.1% Tween 20/TBS solution, filters were incubated with the appropriated peroxidase-conjugated secondary antibodies, washed in 0.1% Tween 20/TBS solution, and developed using the POD chemiluminescence detection system (Roche, Indianapolis, IN). Image analysis and quantifications were performed by using the Kodak Image Station (KDS IS440CF 1.1) with 1D Image Analysis software.

#### *Immunofluorescence analysis*

For immunofluorescence analysis, cells cultured in 35-mm Petri dishes were washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Fixed cells were washed in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked for 1 h in 2% horse serum in PBS and incubated for 1 h at 37°C with mouse monoclonal anti-SOD1 antibody (clone S.D.-G6, Sigma-Aldrich), with rabbit anti-SOD2 (1:400; Stressgen) and with monoclonal anti-cytochrome c antibody (1:400; Promega, Milano, Italy). Cells were washed in blocking buffer and incubated for 1 h with an Alexa Fluor 488 goat anti-mouse (Invitrogen) and Cy3 goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies. After rinsing in PBS, cells were stained with 1  $\mu$ g/ml Hoechst 33342 (Sigma-Aldrich) and examined under a reflected fluorescence microscope (BX51, Olympus, Milano, Italy) equipped with an F-View digital camera and the

Cell-F Digital Imaging Software. Fluorescence images were processed using Adobe Photoshop (Adobe, San Jose, CA).

#### *Cell viability and caspase 3 activity assays*

Cell viability was assessed by colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous one solution assay, Promega), accordingly to manufacturer's instructions. Absorbance at 490 nm was measured in a multilabel counter (Victor3-V, PerkinElmer Life Sciences, Waltham, MA). Caspase 3 activity was measured with a TruePoint Caspase 3 assay kit (PerkinElmer), accordingly to manufacturer's instruction.

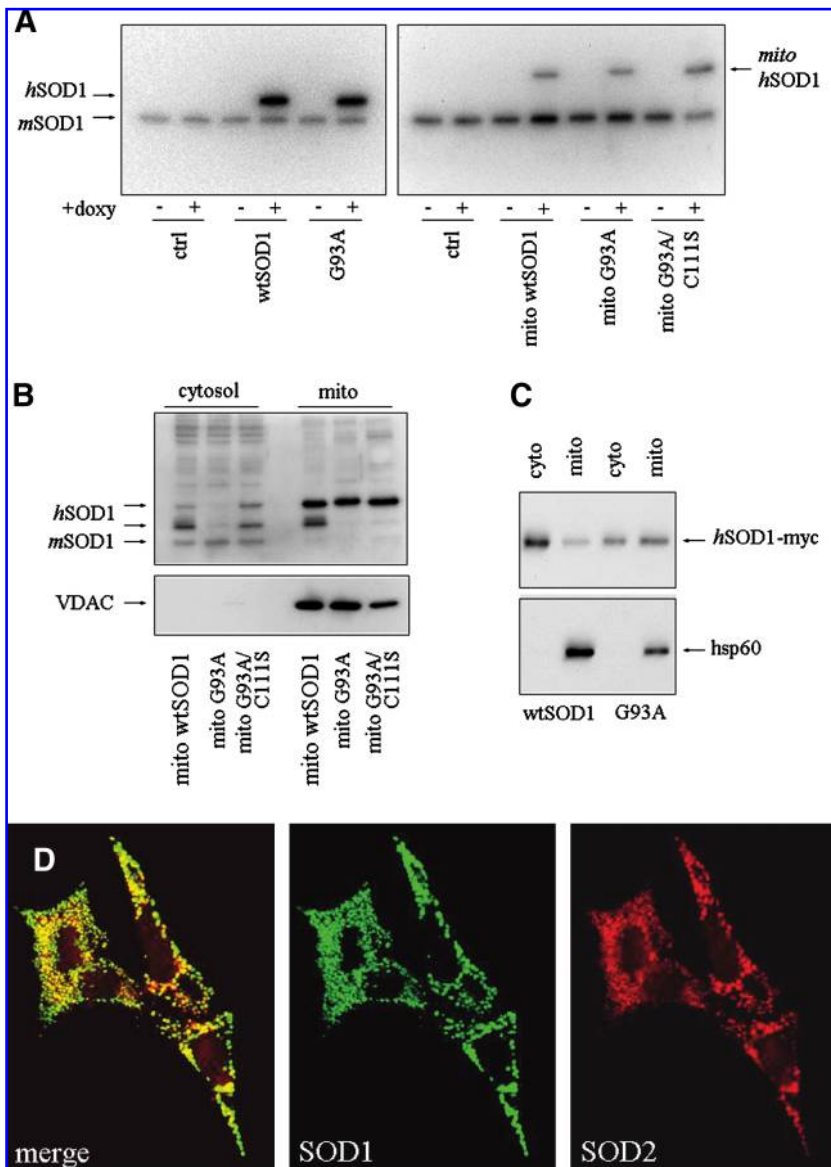
#### *Statistical analysis*

The results are given as means  $\pm$  SD of three independent experiments. Statistical evaluation was conducted by a simple Student's *t* test, and values significantly different from the respective controls are indicated.

### **Results**

#### *The obligatory accumulation of mutSOD1 in mitochondria elicits mitochondrial damage and cell death*

To determine whether the mitochondrial localization of mutSOD1 is crucial to mutSOD1-mediated cell death, we produced stable clones of mouse motoneuronal NSC-34 cells where the mitochondrial accumulation of wild-type SOD1 or mutSOD1s is driven by the 5' leader sequence of mouse cytochrome c1, known to drive cytochrome c1 into the intermembrane space of mitochondria (1). Since the constitutive expression of mitochondrial-targeted mutSOD1 is toxic for cells (unpublished observations), the expression of mitochondria-targeted SOD1s (mito-SOD1s) was put under the control of the pTet-ON inducible vector. Stable cell lines were selected and the inducible expression levels of cytosolic and mitochondria-targeted (mito-SOD1s) wild-type SOD1, the FALS-linked G93A mutSOD1, plus an artificial mito-G93A mutant lacking cysteine 111, were evaluated by Western blot on total protein extracts (Fig. 1A). As expected, mito-SOD1s accumulate almost entirely in the mitochondria of transfected cells (Fig. 1B and D). On the contrary, only a small fraction of SOD1 is present in the mitochondria of cells overexpressing a myc-tagged version of the same proteins (Fig. 1C), in line with previous observations (10), indicating that in mito-SOD1-expressing cells a strong increase in mitochondrial SOD1s is achieved. The expression of mito-G93A-SOD1, but not of wild-type mito-SOD1, induces a significant impairment of mitochondrial metabolism, as assessed by an MTS assay, which becomes evident at 48 h after induction of SOD1 expression and further increases at 72 h (Fig. 2A). Conversely, a modest decrease in the metabolism of mitochondria is recorded in cells expressing G93A-SOD1 only after 72 h. This phenotype is matched by a marked alteration in the morphology of mitochondria, as observed by immunofluorescence analysis of NSC-34 cells using antibodies recognizing SOD1 and SOD2 (MnSOD, a mitochondrial matrix protein). As shown in Fig. 2B, cells expressing the G93A-SOD1 mutant show a significant alteration of the filamentous mitochondrial network which characterizes untransfected or



**FIG. 1. Characterization of mito-SOD1s-expressing NSC-34 cells.** (A) NSC-34-derived cell lines expressing human wtSOD1 or the G93A mutant, and the mitochondria-targeted SOD1s indicated were left untreated (–) or treated (+) for 48 h with 1  $\mu$ g/ml of doxycycline to induce expression of transgenic SOD1. Cell lysates were subjected to SDS-PAGE. The anti-SOD1 antibody used for immunodetection recognizes both human hSOD1s and mouse SOD1 (mSOD1). (B) NSC-34-derived cell lines expressing mitochondria-targeted human wtSOD1 or mutSOD1s as indicated were treated for 48 h with 1  $\mu$ g/ml of doxycycline to induce expression of transgenic SOD1. Equal amounts of lysates from mitochondrial and cytosolic fractions were analyzed in Western blot for SOD1 expression with an anti-SOD1 antibody or with an anti-VDAC antibody to check for the enrichment of mitochondrial fractions. An additional band between mouse SOD1 and hSOD1 was occasionally observed in some experiments, probably due to processing or proteolysis of mitoSOD1s. (C) NSC-34-derived cell lines expressing myc-tagged human wtSOD1 or G93A mutSOD1s were treated for 48 h with 1  $\mu$ g/ml of doxycycline and analyzed as in (A). An antibody recognizing Hsp60 (heat shock protein 60, a mitochondrial matrix protein) was used to check for the enrichment of mitochondrial fractions. 0.5  $\mu$ g of cytosolic proteins were loaded to obtain levels of SOD1s similar to that from 5  $\mu$ g of mitochondria proteins. (D) NSC-34 cells expressing mito-wtSOD1 were subjected to immunofluorescence analysis with anti-SOD1 (green) and anti-SOD2 (red) antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

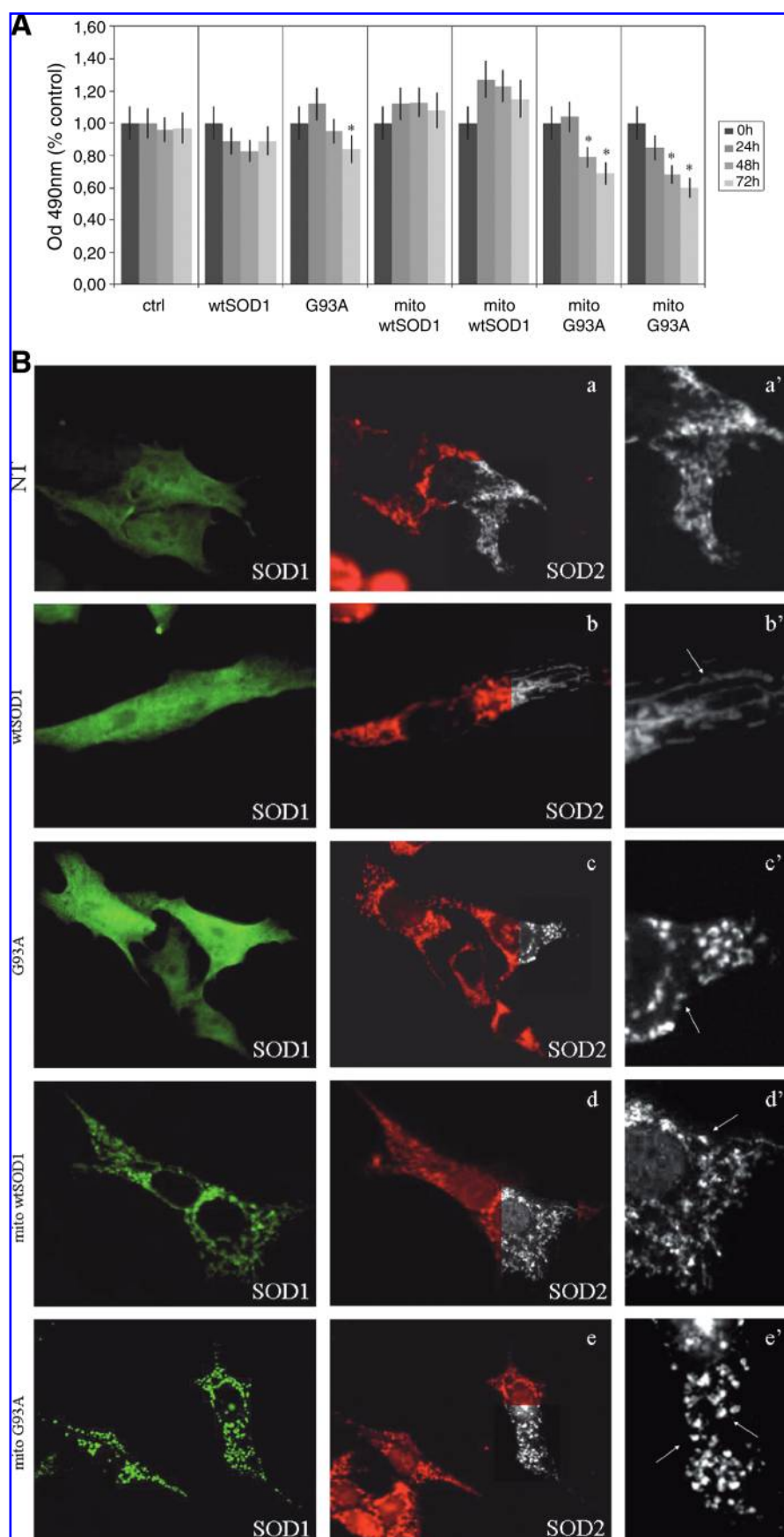
wild-type SOD1-transfected cells, with mitochondria appearing fragmented and swollen. A certain degree of fragmentation is also achieved by mito-wtSOD1, while in cells overexpressing mito-G93A-SOD1 the filamentous network is essentially lost, and mitochondria emerge as swollen and isolated speckles. When human SH-SY5Y neuroblastoma cells are transiently transfected with the same constructs, similar results are obtained, and many nuclei of cells staining positive for SOD1 expression show a clear apoptotic morphology (Fig. 3A). Accordingly, activation of caspase 3 is increased in cells where G93A mutSOD1 is accumulated in mitochondria (Fig. 3B).

#### *A soluble G93A mutSOD1 is less toxic to cells when accumulated in mitochondria*

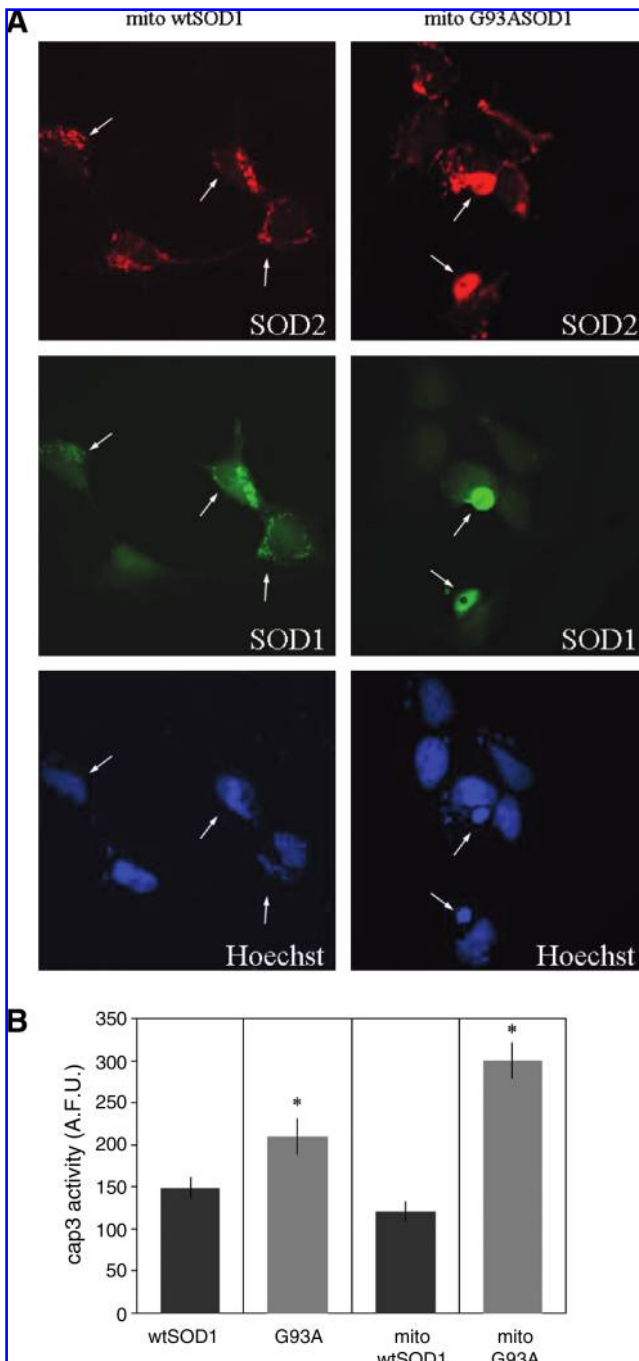
We have recently shown that the toxicity of mutSOD1s is linked to their ability to accumulate in disulfide-linked oligomeric forms that are insoluble in nonionic detergents such as 0.5% NP-40 (5). We therefore analyzed the solubility and

oligomerization potential of wild-type and mutSOD1s that are stably resident in mitochondria because of their mitochondrial localization signal. Figure 4A shows that the behavior of SOD1s and mito-SOD1s are comparable as far as their solubility and oligomerization are concerned, the G93A mutant proteins being much more insoluble and aggregated than their wild-type counterparts. Notably, mito-wtSOD1 appears to be less soluble and more oligomerized than the wild-type enzyme in NSC-34 motoneuronal cells, suggesting a specific contribution of the mitochondrial environment to SOD1 misfolding. The removal of Cys111 is sufficient to shift back to a completely soluble phenotype. Similarly, the mito-G93A/C111S and mito-G93A/C6S/C111S mutants appear in a completely soluble, non-oligomerized form, indicating that extensive disulfide crosslinking mediated by cysteine 111 characterizes mitochondrial SOD1 in cells. Likewise, G93A mutSOD1, but not wtSOD1, accumulates in a detergent-insoluble, disulfide-linked oligomeric form in mitochondria from the spinal cord of transgenic mice in the late stage of the disease course (Fig. 4B).

**FIG. 2. Effects of mito-G93A SOD1 on mitochondrial metabolism and morphology of NSC-34 cells.** (A) NSC-34 cells expressing the indicated cytosolic and mitochondrial SOD1s were treated with 1  $\mu$ g/ml of doxycycline for the indicated periods of time and cell viability was assessed by an MTS assay. Two different clones of mito-wt- and mito-G93A-expressing cells were used. Absorbances at 490 nm are expressed as percent of the relative untreated control cells. Values significantly different from relative controls are indicated with an *asterisk* when  $p < 0.01$  ( $n = 3$ ). (B) After 48 h, the indicated cell lines were subjected to immunofluorescence analysis with anti-SOD1 (green) and anti-SOD2 (red) antibodies. Insets in a', b', c', d', and e' are higher magnifications of areas highlighted in gray scale in a, b, c, d, and e, respectively, to show mitochondrial morphology. NT indicates untransfected NSC-34 cells. Arrows point to fragmented mitochondria. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).







**FIG. 3. Effects of mito-G93A SOD1 on the viability of SH-SY5Y cells.** (A) SH-SY5Y neuroblastoma cells were co-transfected with a pTetOn plasmid together with pTRE2 plasmids coding for wild-type and G93A mitochondria-targeted SOD1s. After a 48 h culture in the presence of 1  $\mu$ g/ml of doxycycline, cells were analyzed by immunofluorescence with anti-SOD1 (green) and anti-SOD2 (red) antibodies. Nuclei of cells were stained with 1  $\mu$ g/ml Hoechst 33342. White arrows point to SOD1-expressing cells. (B) Caspase 3 activity was assessed in cells treated as in (A) and is expressed as arbitrary fluorescence units (A.F.U.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

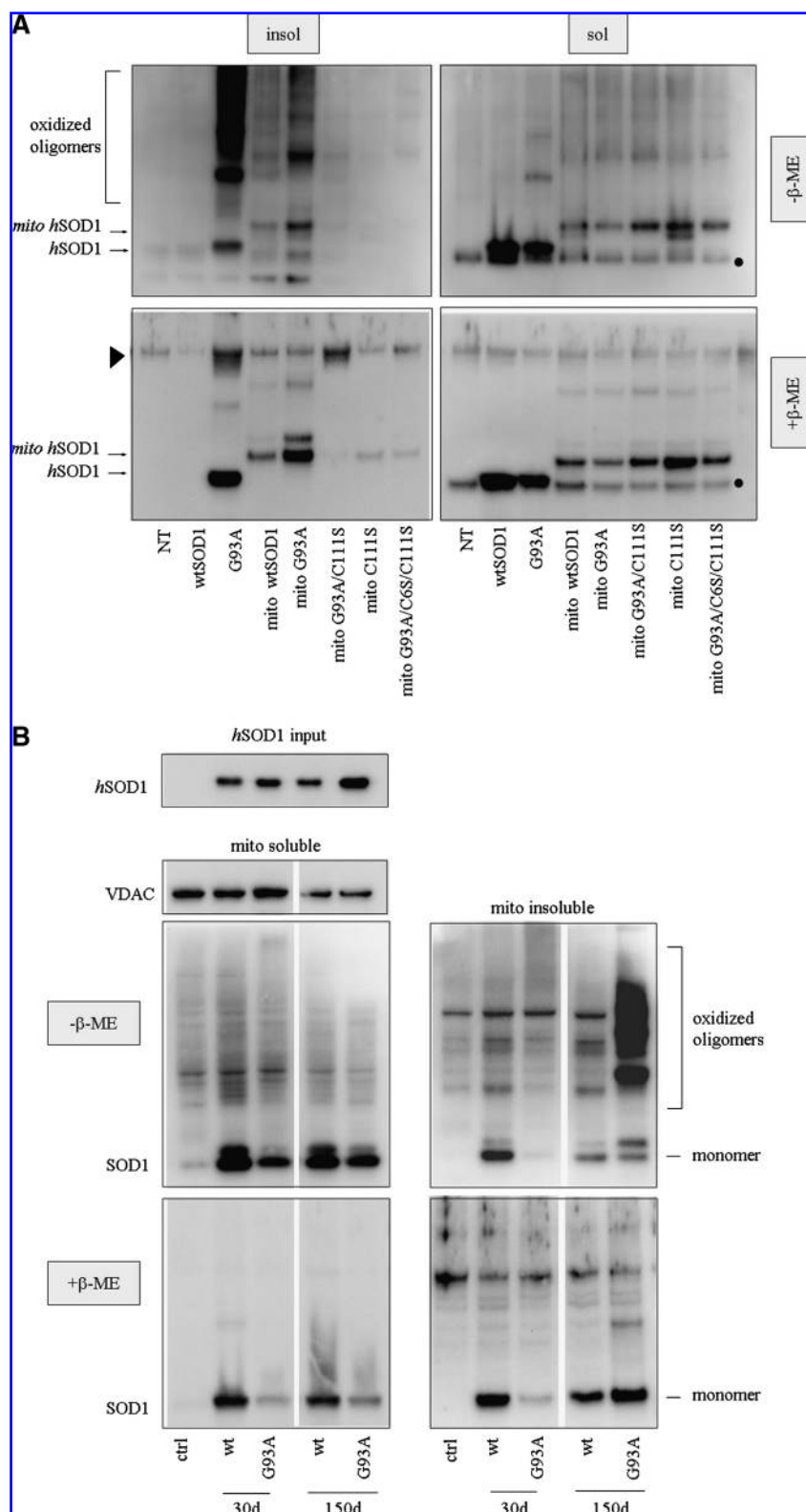
Since a mutant SOD1 where the oxidation of cysteine residues has been impaired by the elimination of cysteine 111 is less toxic when expressed in motoneuronal cells (5), we analyzed the effect of Cys111 removal on the ability of mito-G93A-SOD1 to affect mitochondrial metabolism and cell viability. As reported in Fig. 5, a 96 h expression of the mitochondria-targeted G93A mutant with a replaced Cys111 neither alters the viability of cells (Fig. 5A), nor induces the appearance of an apoptotic phenotype, as assessed by Hoechst staining of cells and caspase activity assay (Fig. 5B and C), despite the overall speckled mitochondrial morphology. On the contrary, apoptosis is achieved in cells expressing the mito-G93A mutSOD1, indicating that the toxicity of mutSOD1s is strictly related to the gaining of a misfolded state in mitochondria of cells. Immunofluorescence analysis with an anti-SOD2 antibody at earlier time points (*i.e.*, 48 h) shows that the overall morphology of mitochondria is still affected by the mito-G93A/C111S-SOD1 (Fig. 6), further suggesting that aggregation of mutSOD1s is not needed for mutSOD1 to induce mitochondrial alterations, and that other forms of misfolded SOD1s may be involved.

#### *CCS overexpression prevents mutSOD1, but not mito-mutSOD1, misfolding.*

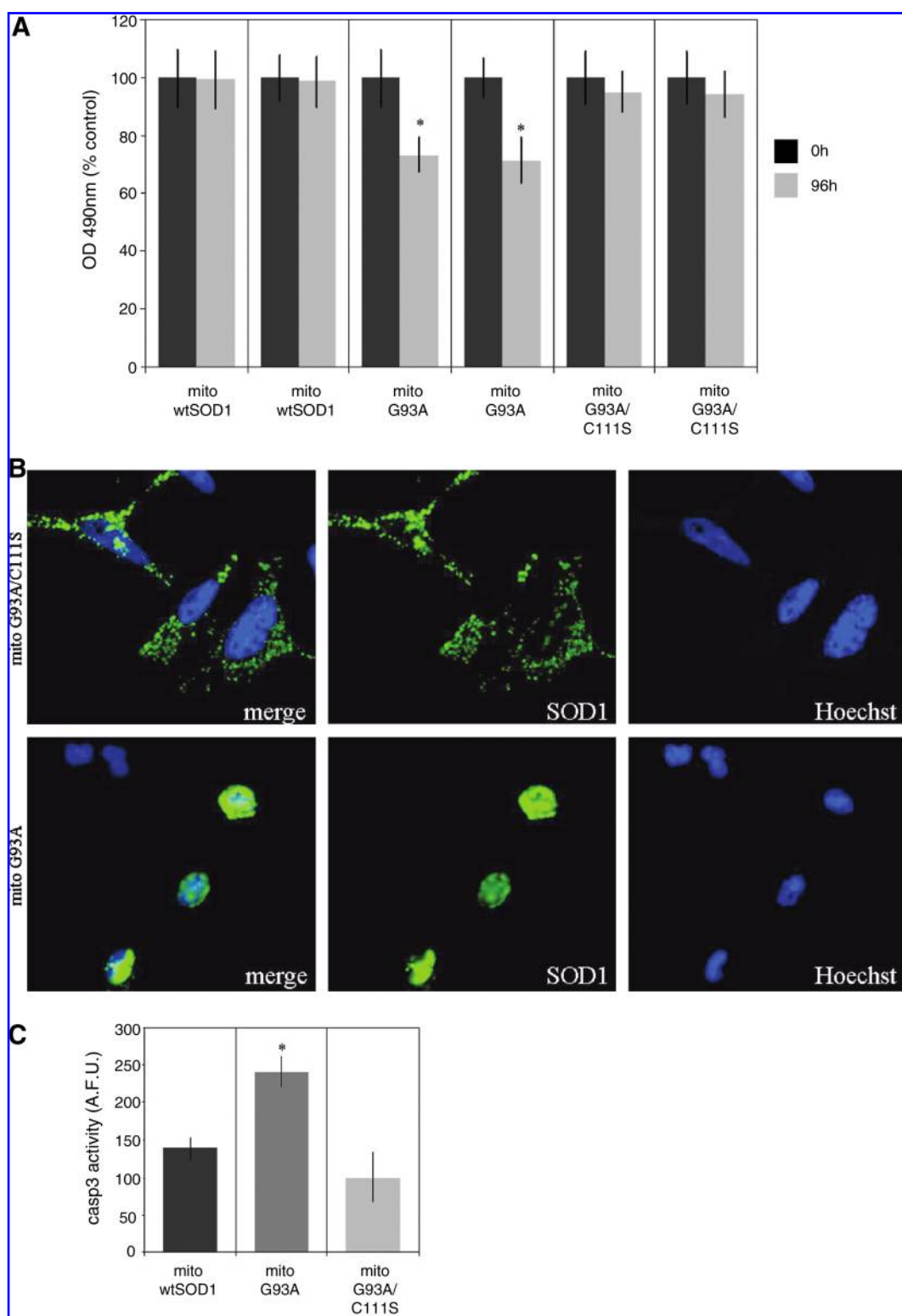
The CCS copper chaperone is critical for both maturation of SOD1 in the cytosol through delivery of the copper co-factor and oxidation of an intra-subunit disulfide between Cys57 and Cys146, and for the recruitment of SOD1 inside mitochondria (7, 24, 30). We therefore asked whether modifying the amount of cellular CCS could influence the folding stability of cytosolic and mitochondrial SOD1s. For these reasons, NSC-34 cells stably overexpressing CCS were produced (Fig. 7). Subcellular fractionation shows that a significant amount of CCS is accumulated in mitochondria of transfected cells, suggesting that under our experimental conditions, CCS is efficiently imported in mitochondria (Fig. 7A). When NSC-34 cells are transfected with plasmids coding for wild-type or mutant SOD1s, the presence of an overexpressed CCS significantly improves the solubility of the FALS-mutant G93A and of mutant G93A proteins lacking one of the cysteines, Cys6 or Cys57, which have been previously shown to be highly insoluble despite the lack of this two cysteines (Fig. 7B). In particular, CCS overexpression enhances the solubility of the G93A mutant even in the absence of Cys57, suggesting that CCS can act on the folding of SOD1 independently from the oxidation of the SOD1 disulfide. On the contrary, the solubility of mitochondria-targeted SOD1 is increased by the overexpression of CCS (Fig. 7C), while Mito-G93A-SOD1 is unaffected or slightly more insoluble and oligomerized when co-expressed with CCS than in cells transfected with a control plasmid. Similarly, in fibroblasts from CCS<sup>-/-</sup> and control CCS<sup>+/+</sup> mice (Fig. 8A), the solubility of G93A mutSOD1 is improved by the presence of CCS, while, in the same conditions, both mito-wtSOD1 and mito-G93A-SOD1 strongly accumulate in a detergent-insoluble form (Fig. 8B).

#### **Discussion**

A small fraction of wtSOD1, as well as of the mutant SOD1s, associates with various mitochondria compartments, namely the intermembrane space and the matrix, and both the inner and outer membranes of spinal cord and brain

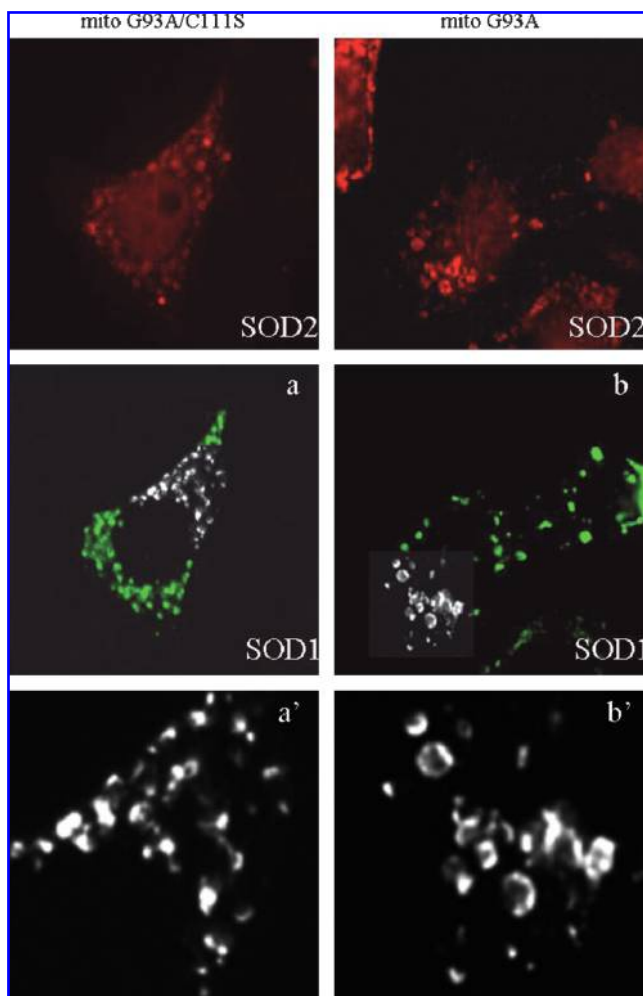


**FIG. 4. Solubility and oligomerization of cytosolic and mitochondrial SOD1s.** (A) Soluble and insoluble fractions were isolated from NSC-34 cells transfected as indicated. Insoluble fractions were solubilized in a buffer containing 2% SDS. Soluble and insoluble fractions were then boiled for 5 min, 100°C, in the presence of 100 mM iodoacetamide. 20  $\mu$ g of proteins from each sample were subjected to a denaturing PAGE, either nonreducing (without  $\beta$ -mercaptoethanol,  $-\beta$ -ME, upper panel) or reducing ( $+\beta$ -ME, lower panel). Western blot analysis was performed with an anti-SOD1 antibody. The black dot indicates the position of mouse, endogenous SOD1 that serves as standard for equal loading of soluble proteins. Black arrowhead points to nonspecific bands that were not seen in other separated experiments. (B) Mitochondria were isolated from control mice or mice transgenic for the expression of wild-type or G93A mutant SOD1 at the indicated age. Soluble and insoluble fractions were analyzed as in (A). 20  $\mu$ g of cytosolic proteins were also analyzed in Western blot with an anti-SOD1 antibody (SOD1 input).



**FIG. 5. Effects of Cys111 removal from mito-G93A SOD1 on mitochondrial metabolism and cell death.** (A) NSC-34 cells expressing the indicated mitochondrial SOD1s were treated with 1  $\mu$ g/ml of doxycycline for 96 h. Cell viability was assessed by an MTS assay. Two different clones of each cell line were used. Absorbances at 490 nm are expressed as percent of the relative untreated control cells. Values significantly different from relative controls are indicated with an *asterisk* when  $p < 0.01$  ( $n = 3$ ). (B) Cells were treated as in (A) and analyzed in immunofluorescence with an anti-SOD1 antibody. Nuclei were stained with Hoechst 33342. (C) Cells treated as in (A) were assayed for the activity of caspase 3. A.F.U: arbitrary fluorescence units. Values significantly different from relative controls are indicated with an *asterisk* when  $p < 0.01$  ( $n = 3$ ). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).



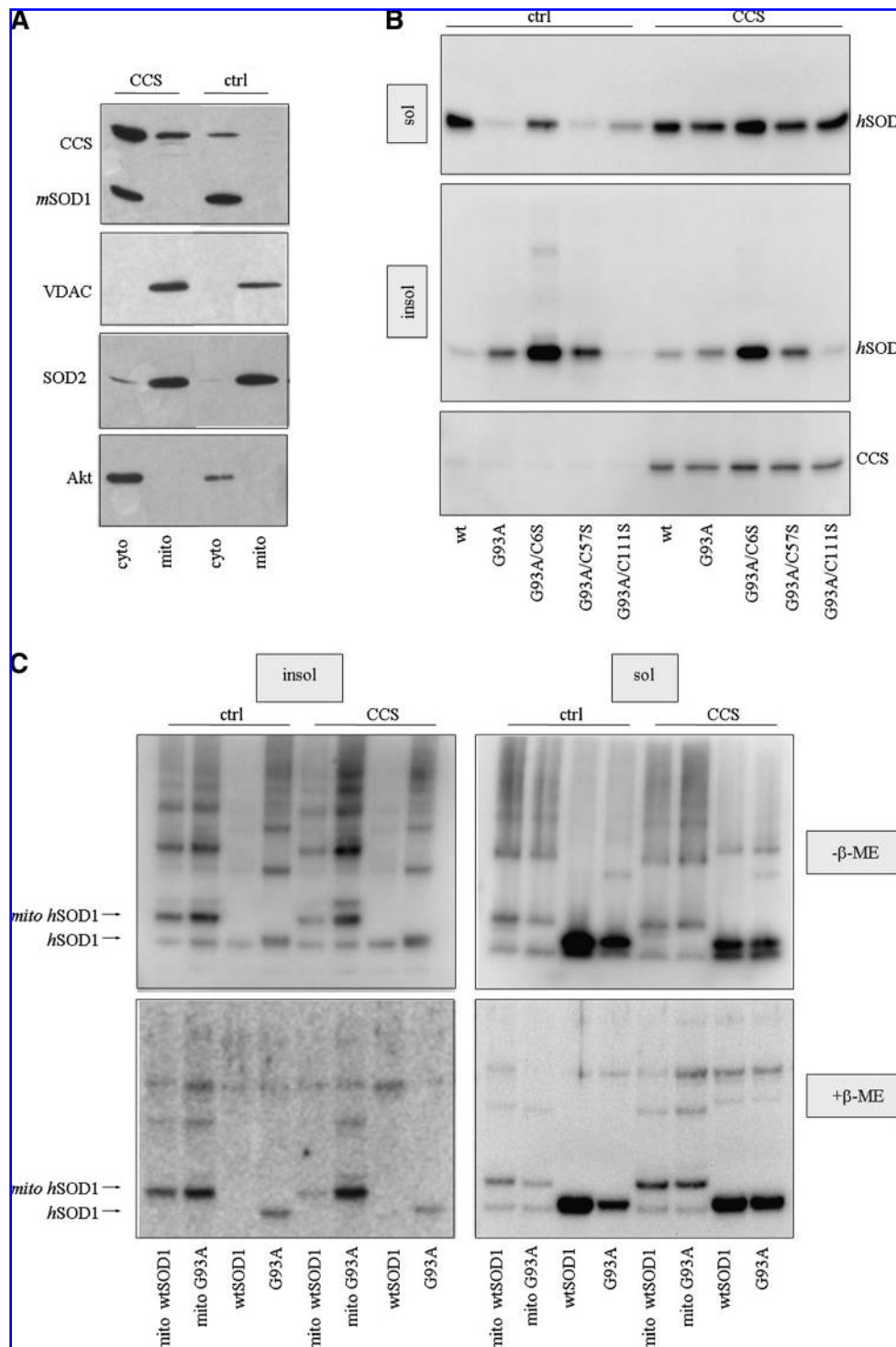


**FIG. 6. Effects of Cys111 removal on mitochondrial morphology.** NSC-34 cells expressing the indicated mitochondrial SOD1s were treated with 1  $\mu$ g/ml of doxycycline. After 48 h of time, cells were subjected to immunofluorescence analysis with anti-SOD1 (green) and anti-SOD2 (red) antibodies. Insets in a' and b' are higher magnifications of areas highlighted in gray scale in a, and b, respectively, to show mitochondrial morphology. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

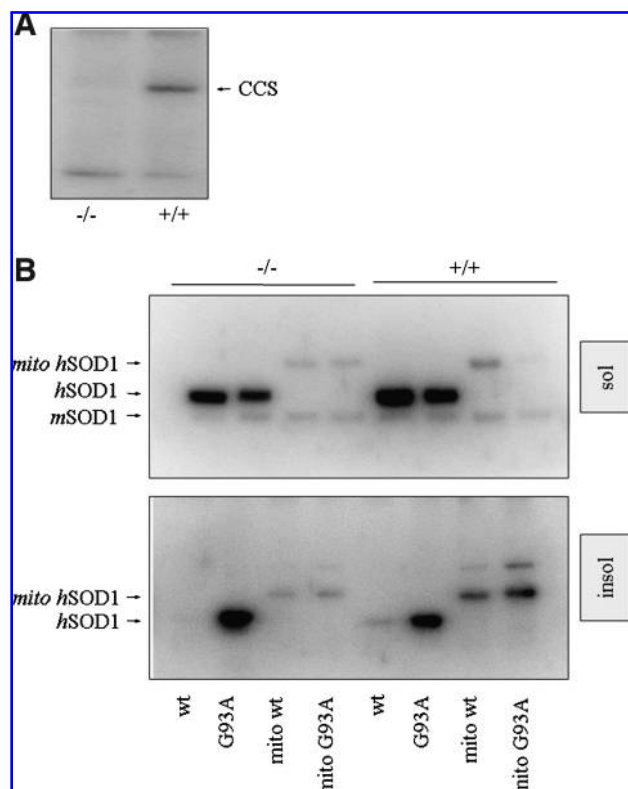
mitochondria (8, 18, 22, 25, 32, 33). This evidence has been considered a clear indication of a direct link between the gained toxic function of mutSOD1 and the alteration of the physiology of mitochondria which is recorded in models and patients suffering from ALS. Although the mechanism of SOD1 accumulation inside mitochondria is still poorly understood, it appears that a complex interaction between respiratory chain activity, oxygen concentration, interactions with CCS, and proper maturation and folding of SOD1 controls its intracellular distribution. It has been suggested that mutant SOD1 escapes this physiological regulation and accumulates in mitochondria due to misfolding and aggregation (21), and this event may be promoted by alterations of the redox environment of mitochondria, such as those induced by inflammatory cytokines, which play a role in ALS as reinforcing signals from glia cells (11).

Emerging evidence attributing to cysteine residues a possible role in mitochondrial localization of SOD1 deserves particular attention. Recently, we and others have reported that removal of Cys111 from a FALS-linked mutSOD1s reduces their accumulation in mitochondria, and this effect is followed by a decrease in the production of oxidative stress and functional impairment of mitochondria that are both induced when mutSOD1s are overexpressed in cells (5, 23). The same cysteine appears to have important roles in the process of mutSOD1s misfolding and aggregation, since the removal of Cys111 from SOD1 mutants is able to make them soluble, and this in turn results in the rescue of cell viability (10, 21). Whether a cysteine-dependent accumulation of mutSOD1s in mitochondria is *per se* sufficient to induce mitochondria impairment, irrespective of the misfolded and aggregated state of the protein, or not, is therefore an issue to be resolved. We reasoned that a SOD1 enzyme that was forced to reside in mitochondria of cells could help in discerning between these two possibilities. Using these mitochondria-targeted SOD1s, we found that the accumulation of G93A mutSOD1 inside mitochondria of cell lines of neuronal origin (NSC-34 and SH-SY5Y) has profound effects on the metabolism and morphology of mitochondria, and overall on cell viability, in agreement with previous reports (31). When overexpressed in mitochondria, mito-wtSOD1 shows a certain degree of insolubility, oxidation, and misfolding, as indicated by the presence of disulfide-linked, high molecular weight oligomers that are absent in cells overexpressing a cytosolic wtSOD1. This suggests that in particular conditions (*i.e.*, when forced into mitochondria), wild-type SOD1 can behave similarly to mutSOD1 and is in line with studies showing that wild-type SOD1 acquires cell death-inducing properties typical of ALS-linked mutant forms through oxidation (9, 27). However, while having some effects on the overall morphology of mitochondria, in the conditions used in this study, mito-wtSOD1 does not affect the viability of cells, indicating that wtSOD1 retains biochemical properties different from mutSOD1s even when targeted to mitochondria and that a threshold for insoluble SOD1 is required to gain a truly toxic function. Apparently, this threshold is achieved by mito-G93A-SOD1, which accumulates in an oligomerized form in mitochondria of both NSC-34 transfected cells and in the spinal cord of diseased G93A mutant mice. Similarly to what has been observed for cytosolic mutSOD1 (5, 19), this effect relies on reactive cysteine 6 and, most of all, cysteine 111. A G93A mutSOD1 that is more soluble by virtue of the removal of Cys111 is less toxic to cells when accumulated in mitochondria. This result strongly indicates that the primary determinant of mutSOD1 toxicity is the acquisition of a misfolded state and not the exclusive accumulation inside mitochondria, although the latter effect may depend upon the former (21). This conclusion is further supported by the observation that decreasing the amount of SOD1 aggregates by introducing a C111S substitution does not result in a proportional decrease of mutSOD1 mitochondrial localization, yet it has strong effects on its toxicity (10, 21).

The nature of the misfolded state of mutSOD1 which is toxic for cells is not yet clear. Our results indicate that it may be represented by disulfide-linked oligomers. However, recent reports propose that disulfide reduced, misfolded monomers may be the toxic form of mutSOD1 (35). This conclusion is mainly sustained by the observation that in mutant mice where CCS is transgenically overexpressed, the pathological phenotype is dramatically accelerated, nonetheless this effect



**FIG. 7. Effects of CCS overexpression on the solubility and digomerization of cytosolic and mitochondrial SOD1s.** (A) Lysates from mitochondrial and cytosolic fractions from NSC-34/pTetON7 cells, both untransfected (ctrl) or stably transfected for the overexpression of CCS, were analyzed in Western blot for CCS and SOD1 expression with anti-CCS and anti-SOD1 antibodies. Anti-VDAC and anti-SOD2 or anti-Akt antibodies were used to check for the purity of mitochondrial and cytosolic fractions, respectively. (B) NSC-34/pTetON cells and NSC-34/pTetON cells stably overexpressing CCS were transfected with pTRE plasmids coding for wtSOD1 or the indicated mutSOD1s. To induce the expression of SOD1, 1  $\mu$ g/ml doxycycline was added. After 48 h of culture, insoluble (insol) and soluble (sol) fractions were isolated as described. Equal volumes from each fraction were subjected to standard reducing SDS-PAGE, and analyzed by Western blotting with an antibody anti-SOD1. Soluble fractions were also analyzed for CCS expression. (C) NSC-34/pTetON7 cells and NSC-34/pTetON7 cells stably overexpressing CCS were transfected with pTRE plasmids coding for the indicated SOD1s. After 48 h of culture in the presence of doxycycline, soluble and insoluble fractions were isolated and subjected to a denaturing PAGE, either nonreducing ( $-\beta$ -ME), or reducing ( $+\beta$ -ME). Western blot analysis was performed with an anti-SOD1 antibody.



**FIG. 8.** Effects of CCS removal on the solubility of cytosolic and mitochondrial SOD1s. (A) Fibroblasts from CCS +/+ or -/- mice were analyzed in Western blot for CCS expression. (B) Fibroblasts from CCS +/+ or -/- mice were co-transfected with the pTetON plasmids together with plasmids coding for cytosolic and mitochondrial wild-type or G93A mutSOD1. After 48 h of culture in the presence of doxycycline, soluble and insoluble fractions were isolated. Equal volumes from each fraction were subjected to standard reducing SDS-PAGE and analyzed by Western blotting with an antibody anti-SOD1.

does not correlate with an enhanced deposition of mutant aggregates that are absent at the time the double transgenic mice die (28). Also, CCS overexpression fails to enhance disulfide oxidation of mutSOD1s both in tissue of mice and, to a lesser extent, in HEK 293 cultured cells, a fact that has been interpreted as to lead to some increase in a pool of disulfide-reduced SOD1 (26). This pathway could be particularly relevant in mitochondria, as we observed that CCS increases the insolubility of mitochondrial mutSOD1, but improves that of cytosolic SOD1s. Since mito-targeted SOD1s are excluded from any influence of CCS-dependant import inside mitochondria, the obvious conclusion is that, in mitochondria, CCS may facilitate the accumulation of a form of mutSOD1 (e.g., disulfide-reduced) that is more susceptible to oxidation and aggregation. In the cytosol, this effect could be not evident because of the possible intervention of cytosolic enzymes, such as glutaredoxins, that could participate with CCS in the folding process of the enzyme (4, 5). Interestingly, G93A/CCS double transgenic mice display a marked mitochondrial pathology, with CCS accumulating in mitochondria driving the increase of mitochondrial SOD1. It would be interesting to verify whether in spinal cord mitochondria of these

mice, an early mitochondria accumulation of aggregated mutSOD1, which is not present in the cytosol, can explain the accelerated death of mice. Finally, it is worth noticing that a soluble mitochondrial mutSOD1, such as the G93A/C111S mutant, still produces clear alterations in the filamentous architecture of mitochondria. Although this is not apparently relevant to the physiology of the transfected cells used in this study, it may suggest that the toxicity of mutSOD1s is not exclusively related to the gaining of an aggregated state, and that other forms of misfolded SOD1s may be involved.

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### Abbreviations

ALS, amyotrophic lateral sclerosis;  $\beta$ -ME,  $\beta$ -mecaptoethanol; CCS, copper chaperone for SOD1; FALS, familial amyotrophic lateral sclerosis; IMS, intermembrane space; mutSOD1, mutant Cu,Zn superoxide dismutase; SALS, sporadic amyotrophic lateral sclerosis; SOD1, Cu,Zn superoxide dismutase; SOD2, Mn superoxide dismutase.

### Author Disclosure Statement

No competing financial interests exist.

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