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PII: S1874-9399(15)00248-5
DOI: doi: 10.1016/j.bbagrm.2015.11.009
Reference: BBAGRM 966

To appear in: *BBA - Gene Regulatory Mechanisms*

Received date: 21 September 2015
Revised date: 19 November 2015
Accepted date: 23 November 2015

Please cite this article as: Michela Dell'Orco, Pamela Milani, Laura Arrigoni, Orietta Pansarasa, Valentina Sardone, Elisa Maffioli, Francesca Polveraccio, Matteo Bordoni, Luca Diamanti, Fiorenzo A. Peverali, Gabriellla Tedeschi, Cristina Cereda, Hydrogen peroxide-dependent oxidative stress induces SOD1 transcription gene is independent from Nrf2 transcription factor in a cellular model of neurodegeneration, *BBA - Gene Regulatory Mechanisms* (2015), doi: 10.1016/j.bbagrm.2015.11.009

Hydrogen peroxide-dependent oxidative stress induces SOD1 transcription gene is independent from Nrf2 transcription factor in a cellular model of neurodegeneration.

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Abstract.

Background. It is still unclear whether oxidative stress (OS) is a disease consequence or is directly involved in the etiology of neurodegenerative disorders (NDs) onset and/or progression; however, many of these conditions are associated with increased levels of oxidation markers and damaged cell components. Previously we demonstrated the accumulation of reactive oxygen species (ROS) and increased *SOD1* gene expression within H_2O_2 SH-SY5Y treated cells recapitulating pathological features of Amyotrophic Lateral Sclerosis (ALS). Since we observed a post-transcriptional regulation of *SOD1* gene in this cellular model of ALS, we investigated the transcriptional regulation of *SOD1* mRNA under oxidative stress (OS). **Results**. In response to H_2O_2 treatment, PolII increased its association to *SOD1* promoter. Electrophoretic mobility shift assays (EMSA) and mass spectrometry analyses on *SOD1* promoter highlighted the formation of a transcriptional complex bound to the ARE sequences. WB analyses showed that in our *in vitro* model, H_2O_2 exposure increases Nrf2 nuclear fraction while IP experiments confirmed its phosphorylation and release from Keap1 inhibition. However, H_2O_2 treatment did not modify Nrf2 binding on *SOD1* promoter, which seems to be regulated by different TFs. **Conclusions.** Although our data suggest that *SOD1* is transcriptionally regulated in response to OS, Nrf2 does not appear to associate with *SOD1* promoter in this model of ALS. Our results open new perspectives in the comprehension of two key antioxidant pathways involved in neurodegeneration**.**

Keywords:

Oxidative stress, Neurodegeneration, Transcriptional regulation, Nrf2, SOD1

Abbreviations:

- Alzheimer's Disease (AD);
- Amyotrophic Lateral Sclerosis (ALS);
- Antioxidant/Electrophile responsive Element (ARE/EpRE);
- Composite Elements (CEs);
- familial Amyotrophic Lateral Sclerosis (fALS);
- Neurodegenerative Disorders (NDs);
- Parkinson's disease (PD);
- Peripheral Blood Mononuclear Cells (PBMCs);
- Reactive Oxygen Species (ROS);
- Sporadic Amyotrophic Lateral Sclerosis (sALS);
- tert-butylhydroquinone (tBHQ);
- Transcription Factor (TF);
- Transcription Start Site (TSS);
- Xenobiotic Responsive Element (XRE).

1. Introduction.

Oxidative stress (OS) is one of the main pathogenic mechanisms observed in neurodegenerative disorders (NDs) such as amyotrophic lateral sclerosis (ALS). Studies on familial (fALS) and sporadic (sALS) ALS patients, as well as in cellular and murine models of SOD1-related ALS, showed a common behaviour of mutant and wild-type SOD1 protein under oxidative stress [1–3]. Elevated levels of oxidative damage to proteins, lipids, DNA and RNA species have been found in *post mortem* tissue from sporadic and SOD1-related familial ALS cases, similar to SOD1 models of ALS [4–9]. Compared to other NDs, increased *SOD1* mRNA expression has been identified as a distinctive feature for ALS [10]. Indeed, we have previously reported an increase in *SOD1* mRNA within nervous areas typically affected in the ALS progression, such as the brain stem, spinal cord and motor cortex, as well as in peripheral blood mononuclear cells (PBMCs) derived from sALS patients compared to healthy controls [10,11].

Recently we demonstrated a post-transcriptional regulation of *SOD1* gene under OS, particularly H_2O_2 treatment selectively leads to *SOD1* mRNA stabilization [12]. Interestingly, increased ,mRNA levels seems to be associate with *de novo* protein synthesis; indeed, we showed increased protein levels in both the OS cellular model of ALS and in motor cortex *post mortem* tissues from sALS patients compared to healthy controls [12]. Variations in gene expression are often result of the synergy between transcriptional and post-transcriptional regulatory mechanisms [13]. An inducible regulatory sequence, the antioxidant/electrophile responsive element (ARE/EpRE), has been identified between -356 and -330 base pairs from the transcription start site (TSS) of the human *SOD1* gene promoter [14]. ARE consensus motif TGACnnnGC has been found in promoter regions of many others cytoprotective genes such as: NAD(P)H:quinone reductase *(NQO1)*, Aldo-KetoReductase (*AKRs*), Sulfotransferase (*SULFs*), Multi-Drug resistance associated protein (*MRP*) [15,16]. An ARE binding transcription factor (TF) previously found to be induced by OS is the nuclear erythroid 2-related factor 2 (Nrf2) [17–19]. Kelch-like ECH-associated protein1 (Keap1) brings Nrf2 into close association with the Cullin E3-ligase complex leading to its ubiquitination and proteosomal

degradation. Oxidation or electrophilic reaction of cysteines in Keap1 frees Nrf2 for translocation to the nucleus where interacts with co-transcription factors to increase transcription of genes containing ARE [20].

Research has shown contrasting results related to activation of the Nrf2 pathway in ALS especially considering its relationship with *SOD1* gene regulation. Most data concern the relationship between Nrf2 and mutated SOD1 (mutSOD1) in mouse or cellular fALS model, but the involvement of SOD1 wild-type (wtSOD1) in Nrf2 pathway in sporadic ALS is understudied. Indeed, Nrf2 activation in astrocytes isolated from transgenic mice over-expressing Nrf2 counteract the mutSOD1 toxic gain of function SOD1-G93A transgenic mice when were co-cultured with non-transgenic motor neurons. This protection is due to Nrf2 induction of glutathione production and secretion by astrocytes [21]. However, to further investigate the role of Nrf2 in ALS, Vargas and colleagues [22] determined the effect of absence of Nrf2 or its restricted overexpression in neurons or type II skeletal muscle fibres on symptoms onset and survival in mutant hSOD1 expressing mice. They did not observe any detrimental effect associated with the lack of Nrf2 in two different mutant hSOD1 animal models of ALS [22]. Furthermore, the mRNA and protein expression of Nrf2 and NQO1, an Nrf2 target, was significantly decreased in NSC-34 cells transfected with human SOD1-G93A gene suggesting that mutated SOD1 disrupts the Nrf2/ARE signalling pathway and reduced the ability of cells to protect against oxidative injury [23].

Starting from these evidences, we aimed at identifying if the previously observed increased levels in *SOD1* mRNA and protein induced by H_2O_2 oxidative stress may be related to the activation of a transcriptional mechanism involving Nrf2, a TF notoriously induced by oxidative stress [20]. Unraveling the role that *SOD1* up-regulation and its mechanism plays in ALS pathology it is of great importance, particularly, considering the recent findings on antisense oligonucleotides (ASOs) against *SOD1* mRNA in ALS treatment [24].

2. Methods.

2.1. Cell culture and treatments.

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle/F12 medium supplemented with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 10 mg/mL streptomycin (PAA Laboratories, Pasching, Austria), at 37°C in an atmosphere of 5% CO_2 and 95% humidity. SH-SY5Y cells were exposed to the solvent (PBS) or to 1 mM H_2O_2, 40 µM tBHQ or vehicle (DMSO) (Sigma-Aldrich, Milan, Italy) for different times as indicated in the results/figures.

Cells viability, after 1mM H_2O_2 treatment, has been assessed by Trypan Blue assay. Briefly, SH-SY5Y cells have been treated for 30, 45, 60 and minutes with hydrogen peroxide; after the treatment cells have been trypsinased and resuspended in 1X PBS. Cells suspension has been mixed with 0.4% and Trypan Blue (Sigma-Aldrich) and counted with the automated cells counter TC20 (BioRad, Milan, Italy) to evaluate the percentage of live cells.

2.2. Western blotting (WB).

Total proteins extract were obtained by cells lysis in cold Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), incubating on ice for 20 minutes and centrifuged at 14 000 rpm for 10 minutes. The supernatant represented the total protein extract. Nuclear and cytoplasmic extracts were prepared according to Schreiber et al. (1989) [25] with slight modifications. Protein contents were determined using bicinchoninic acids (BCA) method (Sigma-Aldrich, Italy). WB analysis was performed by SDS–polyacrylamide gel electrophoresis (PAGE). Extracted proteins were separated on 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane using a liquid transfer apparatus (BioRad). The membranes were treated with a blocking solution containing 5% non-fat dry milk in TBS-T buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween, pH 7.5) for 1hr and incubated overnight with the primary antibodies listed below. Immunoreactivity was detected using the donkey anti-rabbit or anti-mouse (GE Healthcare, Little Chalfont, Buckinghamshire, UK) secondary peroxidase-

conjugated antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence detection kit (ECL Select, GE Healthcare, UK). For subsequent staining, primary and secondary antibodies were removed from the membrane by incubation for 20 min at RT in Stripping Solution (100 mM Mercaptoethanol, 2% SDS, and 62.5 mM Tris/HCl, pH 6.7), washed with TBS-T (3 times ×10 min). Membranes were then processed as described above. Densitometric analysis of the bands was performed using ImageJ software (). Primary antibodies**:** (a) rabbit polyclonal anti-SOD1 (Santa Cruz Biotechnology, Inc., USA); (b) mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology, Inc., USA); (c) mouse monoclonal anti-Keap1 (Santa Cruz Biotechnology, Inc., USA); (d) rabbit monoclonal anti Nrf2 phospho S40 (Abcam,, USA); (e) rabbit polyclonal anti-Nrf2 (20)X, Santa Cruz Biotechnology, Inc., USA).

2.3. Immunocytofluorescence (IF).

SH-SY5Y cells plated on coverslips were exposed to the vehicle (PBS) or 1 mM H_2O_2 for 60 min. After treatment, the medium was removed and cells were rinsed twice with PBS. SH-SY5Y cells were then fixed (15 min at RT) using a solution of 10% paraformaldehyde in PBS. Fixed cells were washed with PBS and treated with a blocking solution containing 5% goat serum in 0.1% Tween-PBS for 1 hr, and incubated overnight at 4°C with the primary antibodies: rabbit polyclonal anti-Nrf2 (20)X,-Santa Cruz Biotechnology, Inc., USA). Cells were then washed with PBS, and incubated with secondary antibody (Alexa 488 goat anti-rabbit, Invitrogen, USA) for 1 hr at RT. Samples were finally washed with PBS and incubated for 30 sec with ProLong Gold (Life Technologies), dried for 2 hr and sealed. Nrf2 localization has been detected using a fluorescent microscope (AXIO Imager.M2 Microscope ZEISS) and analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

2.4. Electrophoretic Mobility Shift Assay (EMSA).

Probes were purchased from Eurofins MWG Operon (Milan, Italy). Primer pairs flank the ARE element, the TSS in *SOD1* promoter and two intronic regions.

Probe sequences: *SOD1*-ARE probe IRDye®700-labeled at both 5'-terminus: 5'-CTC AGT CAT AAC TAA TGA CAT TTC TAG A-3' (Fw); 5'-TCT AGA AAT GTC ATT AGT TAT GAC TGA G-3' (Rw). Cold probe containing a canonical ARE sequence: 5'- GTC ACA GTG ACT CAG CAG AAT CTG-3' (Fw); 5'-CAG TGT CAC TGA GTC GTC TTA GAC-3'. 4 µg of nuclear extract from SH-SY5Y cells treated with 1 mM H_2O_2 for 30 and 60 min were incubated with 1 µl of 100nM DNA probe IRDye ®700 (LI-COR Bioscience, Milan, Italy) in EMSA Binding Buffer 1X (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 2,5 mM DTT/Tween-20 2,5%) and 1 µl of 10 µM of cold DNA probe for the competitive assay. Reaction mixtures were incubated for 25 min at RT in the dark and loaded on a non-denaturating 4% polyacrylamide gel (37.5:1 ratio of acrylamide to bis-acrylamide) in 0.5X Tris/borate/EDTA buffer. Samples were resolved at 80V (constant voltage) for 60 min at 4°C in the dark. Probe signals were detected and analyzed using the Odyssey® Infrared Imaging System (LI-COR Bioscience, Italy).

2.5. Streptavidin-agarose pull-down assay.

The analysis of protein-DNA binding by streptavidin-agarose pull-down has been performed as previously described [26] with some modifications. In particular, to generate double-strands biotinylated probes an equal amount of sense and antisense oligonucleotide have been placed for 1hr in a water bath at 100 C. 40 µl of streptavidin-agarose bead suspension was added to a mixture of 200 µg of cellular nuclear extract from SH-SY5Y cells treated for 30 and 60 min with 1 mM H_2O_2 and 4 µg of double-strand biotinylated oligonucleotides in 400 µL of PBSI buffer (PBS buffer plus 1mM DTT and protease inhibitor cocktails). The mixture has been placed on a rocking platform at RT for 2 hrs and centrifuged at 550 g for 1min. The pellet was washed with cold PBSI for three times for 10 min and suspended in 40 µl of 2X Laemmli sample buffer. After the incubation at 95 C for 10 min, samples have been centrifuged at 7000 g for 30s and the collected supernatant load on a 10% SDS-PAGE gel run at 100V for 1hr. The bands relative to electrophoretic separated proteins have been analyzed through Mass Spectrometry after their detection by Silver staining. Probe sequences: *SOD1*-ARE: 5'-CTC AGT CAT AAC TAA TGA CAT TT CTAG A-3; *NQO1*-ARE used as positive control: 5'-

AAT CGC AGT CAC AGT GAC TCA GCA GAA TCT GAG CCT AGG G-3; mutated *SOD1*-ARE used as negative control: 5'-GAT CCT CAG TCA TAA CTA AGT CAG ACT CTA GA-3' [26].

2.6. Mass Spectrometry analysis (MS/MS).

Bands relative to electrophoretic separated proteins were digested *in situ* by trypsin. In particular, upon SDS-PAGE, each band was excised, cut in small pieces, and dried in a Speed Vac. Gel pieces were rehydrated with trypsin solution (sequence grade, Sigma-Aldrich, Italy; 0.3 µg trypsin/band in 100 µl 50 mM ammonium bicarbonate, 9% acetonitrile) and incubated overnight at 37°C. Peptides were extracted from the gel using 0.1% trifluoroacetic acid: acetonitrile (1:1). The material was dried, resuspended in 15 µL 0.3% v/v formic acid and desalted using Zip-Tip C18 (Millipore, Darmstadt, Germany) before mass spectrometric (MS) analysis. Each sample was separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific Inc., USA). Buffer A was 0.1% vol/vol formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 lm ID, 3 lm resin, Dionex). The gradient was as follows: 5% buffer B (10 minutes), 5% to 40% B (60 minutes), 40% to 50% B (10 minutes), 95% B (5 minutes) at a flow rate of 0.3 µL/min. Mass spectrometry was performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific Inc., USA) equipped with a nanospray source (Proxeon Biosystems, now, Thermo Fisher Scientific Inc., USA). Eluted peptides were directly electrosprayed into the mass spectrometer through a standard non-coated silica tip (New Objective, Woburn, MA, USA) using a spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350‑2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific Inc., USA). Protein identification was performed using the Sequest search engine contained in the Proteome Discoverer 1.1 software (Thermo Fisher Scientific Inc., USA). The following parameters were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance, carbamidomethylation of

Cys as fixed modification, Met oxidation, Ser-Tyr-Thr phosphorylation, Lys acetylation as variable modifications, trypsin (2 misses) as protease, False Discovery Rate for peptides 1% (against decoy). Only peptides with Xcorr > 1.0 were included for identification.

2.7. Quantitative real-time reverse-transcription polymerase chain reaction.

Total RNA from SH-SY5Y cells was extracted with Trizol® (Invitrogen, Italy) according to the manufacturer's recommendations. RNA quality and quantity was determined using NanoDrop spectrophotometer (Celbio, Milan, Italy) and 1 µg was reverse transcribed using the iScriptcDNA synthesis kit (BioRad, Italy) following the manufacturer's protocol. PCR amplifications were carried out with the iCycler PCR Detection System using iQSupermix (BioRad, Italy). *GAPDH* gene was used as housekeeping gene (hkg) to normalize values. Primer sequences: *SOD1* Fw: 5'-GGT CCT CAC TTT AAT CCT CTA TCC AG-3', Rv: 5'-CCA ACA TGC CTC TCT TCA TCC-3'; *NQO1* Fw: 5'-GAA CTT CAA TCC CAT CAT TTC CAG-3', Rv: 5'-CAG CTT CTT TTG TTC AGC CAC AAT-3'; Nrf2 Fw: 5'- GTC ACT TGT TCC TGA TAT TCC-3', Rv: 5'-GGA GCT ATT ATC CAT TCC TGA-3'; *GAPDH* Fw: 5'-CAG CAA GAG CAC AAG AGG AAG-3', Rv: 5'-CAA CTG TGA GGA GGG GAG ATT-3'.

2.8. Immunoprecipitation (IP).

Immunoprecipitation was performed according to a previously published protocol with minor modifications [14]: 50µg of proteins diluted in the NT2 buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM MgCl2; 0.05% Nonidet P-40) plus protease inhibitors, 1 mM DTT, 20 mM EDTA were incubated at RT for 2 hrs with 1µg of anti-Keap1 antibody (sc-365626, Santa Cruz Biotechnology, Inc., CA, USA) and with 50µl of protein A/G plus agarose (Santa Cruz Biotechnology, Inc., USA). Samples subsequently were loaded on a WB to be analyzed.

2.9. Chromatin immunoprecipitation (ChIP).

SH-SY5 cells were crosslinked in 1% formaldehyde for 10 minutes at RT and quenched by 5 min incubation with 0.125 M glycine. Plates were rinsed two times in ice-cold PBS and cells were scraped off. Cell pellet volume was measured and resuspended in 10X pellet volumes of hypotonic Swelling Buffer (25 mM HEPES pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.5 % NP-40, and 1 mM DTT, supplemented with Protease inhibitor cocktail). After 10 min incubation on ice, nuclei were resuspended in 12X volumes of Sonication Buffer (50 mM HEPES pH 7.8, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, supplemented with Protease inhibitor cocktail), and chromatin was sheared by sonication to an average DNA size of 200-400 bp. About 15-20 µg of DNA were used in each ChIP reaction and immunoprecipitations were carried out by incubation with 2 µg of RNA pol II 8WG16 monoclonal antibody (USAMMS-126R, Covance, Princeton, NJ, USA), 2 µg of Nrf2 (sc-722 X, Santa Cruz Biotechnology, Inc., USA) and a beads-only control. Each ChIP reaction was incubated at 4 °C O/N on a rotating wheel. 50 µL of saturated and pre-equilibrated A/G agarose beads (sc2300, Santa Cruz Biotechnology, Inc., USA) were added to each ChIP reaction to retrieve antibody-protein-DNA complexes, and then washed six times with the following buffers: two times with Sonication Buffer, two times with Wash Buffer (50 mM HEPES pH 7.8, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, supplemented with protease inhibitor cocktail) and finally two times with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Protein-DNA complexes were eluted with 200 µL of elution buffer (10 mM Tris-HCl pH 8.0, 1 % SDS, 1 mM EDTA) by 20 min incubation at 65 °C following an RNase and proteinase K treatment. The decrosslinked DNA was purified with QIAquick PCR Purification Kit (Qiagen, Venlo, Limburg, The Netherlands) accordingly the manufacturer's instructions. ChIP enrichment was assayed in real time PCR (QuantiTect SyBR Green PCR mix, Qiagen; Roche LightCycler 480 instrument) by analyzing ChIP and input samples in triplicate. Absolute quantification of samples was performed against a standard curve to determine concentration values, and then % of input was calculated for each primer set tested. ChIP primers sequences: Primer pair #1 (ARE elements in *SOD1*

promoter) Fw: 5'-CAA ATG AGA CGC TGT GGC CAA ACT-3', Rv: 5'-GGT TGC AGT ACG CGA AAT GGC A-3'. Primer pair #2 (Transcription start site TSS of *SOD1* gene) Fw: 5'-TCT GGC CTA TAA AGT AGT CGC GGA-3', Rv: 5'-TGC TCG AAA TTG ATG ATG CCC TGC-3'. Primer pair #3 (*SOD1* Intron 1) Fw: 5'-TCT AGG TCA GGG AGT CTT CGC TTT-3', Rv: 5'-TCC CTG CAA AGT GTC ACA CAA ACG-3'. Primer pair #4 (*SOD1* Intron 2) Fw. 5'-AGG CTC CAT TGA TCC TCA TGC CTT-3', Rv: 5'-ATA GAC CAC TTG AGC CCA GCA GTT-3'. Primer pair #5 (*HMOX1* exon 3, NEGATIVE CONTROL) Fw: 5'-CAC CCG CTA CCT GGG TGA C-3', Rv: 5'-GGA GCG GTA GAG CTG CTT GA-3'. Primer pair #6 (ARE elements in the *NQO1* promoter, POSITIVE CONTROL) Fw: 5'-AAG TGT GTT GTA TGG GCC CC-3', Rv: 5'-TCG TCC CAA GAG AGT CCA GG-3'. Primer pair #7 (Transcription start site TSS of *NQO1* gene) Fw: 5'-TTT GCA GCA CTC ACC GAC CAT-3', Rv: 5'- ACG GGC CGG ACA GGA TAT ATA AGA-3'.

2.10. Data analysis.

All experiments were performed at least three times. Values were expressed as means ± S.E.M. Statistical analysis was performed using GraphPad Prism version 5 (La Jolla, CA, USA). The data were analyzed by Student *t*-test and analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test. Differences were considered statistically significant when p values were < 0.05.

3. Results.

3.1. Oxidative stress increases PolII association to *SOD1* promoter.

We analyzed RNA polymerase II (PolII) binding to *SOD1* promoter to assess whether oxidative stress (1 mM H_2O_2) could modulate the expression of this gene at the transcriptional level. To this purpose, we performed chromatin immunoprecipitation followed by quantitative real time PCR (ChIP-qPCR). We detected high PolII association to the region encompassing the TSS and we observed significant increase recruitment after 30, 45 and 60 min of treatment (Figure 1A-1B, primer pair #2; ***p value < 0.001). Primer pairs flank the ARE element, the TSS in *SOD1* promoter and two intronic regions (Figure 1B).

3.2. Multiple TFs and/or co-factors bind ARE sequence on *SOD1* promoter *in vitro* experiments.

To test if *SOD1* mRNA upregulation is mediated by OS-activated transcriptional factors, we first performed EMSA using a probe containing the ARE element on *SOD1* promoter. In Figure 2A we compared the *SOD1*-ARE element to the canonical ARE sequence present on the *NQO1* gene promoter, a noted target of Nrf2 [27]. We found that the ARE sequence in the terminal portion of the *SOD1* promoter contains a GC to TT base pair substitution in respect to the *NQO1* promoter (Figure 2A), suggesting that the *SOD1*-ARE deviate from this more canonical ARE promoter.

As shown in Figure 2B, EMSA highlighted the presence of a dynamic protein-DNA complex represented by three bands (indicated by the arrows) which were differentially shifted by OS. Interestingly, while the intensity of the middle band was unchanged after stimulation, the signal of the upper band decreased after H_2O_2 treatment, and we observed a significant increased signal for the lower band at 30 and 60 min respectively (**p value < 0.01; ***p value < 0.001). The increased signal of the lower band, correlates with increased *SOD1* mRNA at these time points [12] and competes with a canonical ARE-sequence (Supplementary Figure 1), suggesting the formation of ARE-specific mediated complex induced by oxidative stress.

3.3. Relationship between *SOD1* increased transcription and Nrf2 pathway activation.

To test whether Nrf2 binds *SOD1* promoter in the chromatin milieu, we performed Nrf2 ChIP-qPCR experiments in our cellular model. We treated SH-SY5Y cells with either vehicle (PBS) or 1 mM H_2O_2 for 30, 45 and 60 min. Surprisingly, no significant changes in the binding between Nrf2 and the ARE sequence in *SOD1* promoter were detected after H_2O_2 treatment (Figure 3). These results suggested us to employ two positive controls: first, the well-established upregulation under H_2O_2 of the *NQO1* gene, a well-documented target gene of Nrf2 [27]; second we used tert-butylhydroquinone (tBHQ), a strong antioxidant inducer of Nrf2 pathway [28] to investigate Nrf2 activation on *SOD1* promoter. The primer pairs "Nrf2 positive CTR" and "PolII positive CTR" flank the ARE elements and the TSS in *NQO1* promoter respectively; while the negative control primer pair "Negative CTR" amplifies a portion of *HMOX1* exon 3 (Figure 4A, right table). H_2O_2 treatment induced a faint association of Nrf2 to *NQO1* gene promoter demonstrating a weak inducible behavior (t45 **p value < 0.01; Figure 4A). Beside, to verify whether Nrf2 activity on *SOD1* promoter could be treatment specific, we treated SH-SY5Y cells with tBHQ (Figure 4B). As expected, SH-SY5Y exposure to 40 µM tBHQ was associated with Nrf2 translocation to the nuclear compartment (*p value < 0.05; Supplementary Figure 2A) and strong up-regulation of *NQO1* mRNA level (Supplementary Figure 2B; p value < 0.001). By contrast, minimal changes were detected for *SOD1* transcript (Supplementary Figure 2B). Indeed, ChIP-qPCR experiments showed that PolII enrichment at *SOD1* TSS was unmodified after tBHQ treatment (Figure 4B, left graph and Figure 1B, left table) and no significant Nrf2 bounding to *SOD1* promoter upon the exposure to tBHQ (Figure 4B, right graph), while the binding between PolII and Nrf2 on *NQO1* promoter is strongly induced by tBHQ (Figure 4B, left graph).

3.4. Activation of Nrf2 pathway upon H_2O_2 exposure.

In order to verify H_2O_2 OS induced activation of the Nrf2 pathway, we tested phosphorylation of Nrf2 (p-Nrf2) on Ser40 by PKCδ, one of the most common mechanisms of Nrf2 activation. Phosphorylation on this residue causes the release of Nrf2 from Keap1, leading to increased ARE-

dependent gene expression [29,30]. WB experiments revealed a significant increase in p-Nrf2 levels in the nuclear fraction after 60 min (**p value < 0.01) and in cytoplasmic compartment after 30 and 60 min (*p value < 0.05; **p value < 0.01) of H_2O_2 treatment (Figure 5A). Interestingly, total Nrf2 is significantly increased specifically in the nuclear compartment, without alterations in the cytoplasm, suggesting an increased ratio of the activated TF after hydrogen peroxide treatment (Supplementary Figure 3). Additionally, IP experiments in the cytoplasmic fraction showed both a significant decrease in the association between the p-Nrf2 and Keap1 after 60 minutes of treatment (**p value < 0.01; Figure 5B). The p-Nrf2 translocation from cytoplasmic milieu to nucleus has been confirmed by IF localization experiments after 60 min of exposure (Figure 5C). These findings clearly demonstrated that H_2O_2 treatment induced Nrf2 phosphorylation and dissociation from Keap1, leading to its accumulation in nuclear compartment activating the transcriptional pathway.

Finally we evaluate both *Nrf2* and *NQO1* gene expressions under H_2O_2 oxidative stress. We observed significantly reduced levels of *Nrf2* mRNA after 60 min of H_2O_2 treatment (*p value < 0.05; Figure 6), comparable with findings in *post mortem* tissues from ALS patients and mtSOD1 models [31]. On the other hand, we did not find *NQO1* transcriptional induction under oxidative stress (Figure 6) suggesting that hydrogen peroxide may be unable to activate transcription of the cytoprotective genes.

3.5. Nrf2 DNA pull-down assay coupled with Mass Spectrometry on SOD1 promoter.

Finally, in order to identify the TFs involved in orchestrating *SOD1* up-regulation under oxidative stress, we performed a DNA pull-down assay coupled with Mass Spectrometry (MS/MS). Specifically, we employed a probe with the same sequence as the oligonucleotide used for EMSA and a mutated *SOD1*-ARE as negative control. We confirmed the presence of the dynamic protein-DNA complex (Table 1), indeed at t0, t30 and t60, Nrf2 occupies, with significant sequence coverage, *SOD1* promoter along with Aryl Hydrocarbon Receptor (AHR) and Nuclear Factor NF-kB p105 subunit, both known to regulate oxidative stress response in neuronal cells [32–35]. We carried out a

bioinformatics analysis of *SOD1* promoter from the TSS (+1), encompassing the ARE sequences (located between -356 and -330) to -2000 bp upstream the TSS region. We employed an array of predictive software such as TRANSFAC, JASPAR and MatrixCatch 2.7 TRANSFAC® algorithm [36,37] to compare results with data from the literature verifying TFs known to be ROS activated or identified by precedent ChIP-seq experiment. This type of analysis was aimed to identify different TFs likely involved in *SOD1* regulation and to search potential composite elements (CEs) in *SOD1* promoter (Supplementary Table 1).

4. Discussion.

Increased levels of both *SOD1* mRNA and protein have been found in nervous system tissues typically affected by ALS disease (i.e. brain stem, spinal cord and motor cortex) and in PBMCs from sALS patients, hence representing a fundamental and distinctive biomarker for sporadic ALS [10–12]. Therefore, the comprehension of the mechanisms responsible for *SOD1* overexpression constitutes an essential starting point to therapeutically counteract ALS progression.

According to our data, human neuroblastoma cells treated with hydrogen peroxide represent a valid *in vitro* model for the study molecular mechanisms involved in sporadic ALS pathogenesis. In H_2O_2-treated cells we confirmed increased ROS, *SOD1* mRNA overexpression [12] and the formation of high-molecular weight SOD1 aggregates (Supplementary Figure 5). In a study utilizing the SH-SY5Y cell line treated with H_2O_2, and PBMCs obtained from sALS patients, we recently demonstrated that ELAV proteins are involved in the post-transcriptional regulation of *SOD*1 under oxidative stress, through *SOD*1 mRNA stabilization and new protein synthesis [12]. To verify if the *SOD1* overexpression could also be related to transcriptional regulation, we studied the relationship between the Nrf2 transcription factor and *SOD1* promoter. Indeed, it is widely accepted that Nrf2 is a key regulator of the cellular response to oxidative stress by up-regulating detoxification enzymes and antioxidant proteins [17]. The activated Nrf2 translocates to the nucleus where it binds to ARE sequences and activates the expression of different cytoprotective target genes. Moreover, an ARE binding element targeted by Nrf2 has been identified in *SOD1* promoter [14].

By performing PolII chromatin immunoprecipitation and by scanning *SOD1* proximal promoter by real time PCR of co-immunoprecipitated DNA, we reported an increased association of PolII at *SOD1* TSS region after H_2O_2 oxidative stimulation. Since PolII access to gene promoter is required to increase transcription, this data suggested that *de novo* synthesis of *SOD1* mRNA occurs in our cellular model under H_2O_2 treatment. An EMSA assay, performed using a probe harbouring the *SOD1* ARE element, revealed the presence of a dynamic protein-DNA complex. We observed three bands which shifted with differential trends, probably corresponding to distinct protein-DNA complexes.

These results suggested that after H_2O_2 treatment the cis-acting ARE sequence in *SOD1* promoter can exert a critical role by recruiting multiple TFs and/or cofactors, potentially co-operating to fine-tune modulate *SOD1* mRNA induction under oxidative stress. Moreover these findings shed new light on *SOD1* expression under oxidative stress condition, demonstrating H_2O_2-dependent transcriptional induction. Indeed tBHQ, antioxidant compound, is unable to induce increased *SOD1* mRNA expression, supporting the relationship between the increase *SOD1* gene translation and oxidant inducers.

Next, we tried to unravel if Nrf2 played a key role in the complex protein-DNA observed by in the EMSA assay, by performing a Nrf2 ChIP assay followed by a qRT-PCR with primers designed to flank the ARE element in *SOD1* promoter. Surprisingly, Nrf2 occupancy at the ARE elements in *SOD1* promoter did not change after H_2O_2 oxidative treatment. On the other hand we reported a marginally increased association at the ARE element in *NQO1* gene, a well-known Nrf2 gene target. These data indicated that Nrf2 it is not able to gain access to *SOD1* 5'-flanking region in the native chromatin context. Additionally, we found that H_2O_2 treatment did not induced *NQO1* mRNA synthesis suggesting the existence of two different pathways activated by specific compounds. *SOD1* mRNA up-regulation in response to H_2O_2 oxidative treatment might indeed be ascribed to the involvement of other transcriptional factors. We then reported Nrf2 activation through TF phosphorylation on Ser40, its release from Keap1 inhibitor and its enhanced nuclear accumulation. These results confirm that, even if, the main Nrf2 activators are electrophilic agents [38], H_2O_2 induced oxidative stress activates Nrf2. However, at the chromatin level, it seems to regulate a different pathway compared to the antioxidant tBHQ. These data led us to hypothesize that the *SOD1* promoter is not a canonical downstream target of the Nrf2 regulatory network and that Nrf2 transcriptional gene pathway is strictly related to the treatment.

A pull down assay followed by MS/MS further confirmed the presence of the dynamic protein-DNA complex. Indeed in untreated cells and at 30, 60 min of treatment, Nrf2 occupies *SOD1* promoter

along with Thyroid Hormone Receptor beta (THRbeta), Aryl Hydrocarbon Receptor (AHR) and Nuclear Factor NF-kB p105 subunit.

Thus, we hypothesized that Nrf2 may act as a "constitutive" TF binding to *SOD1* ARE sequences even without stimulus, while the H_2O_2 treatment could induce a variation of the chromatin context conformation that induces the *SOD1* gene transcription modifying the relationship between the different identified TFs. Cofactors may be able to mask the presence of Nrf2 on the ARE sequence not allowing the interaction with the anti-Nrf2 antibody in ChIP experiments. Effectively, *SOD1* gene has been erroneously considered a housekeeping gene; nevertheless its inducible transcription is finely regulated by *cis*-acting sequences and the corresponding *trans*-active factors.

With these results, we can conclude that the increase in *SOD1* gene expression induced by H_2O_2 treatment is an intricate process related not only to the activation of a transcriptional mechanism involving PolII, but also other mechanisms such as chromatin conformational changes and epigenetic variations, acting in concert to modulate the rate between *de novo* synthesis, mRNA stabilization, and protein stabilization. Undoubtedly, further studies will be necessary to identify the TFs directly involved in *SOD1* transcriptional induction under H_2O_2 oxidative stress, and unravel the implication of the other mechanisms involved in SOD1 transcription pathway. It is of great importance to clearly understand the mechanism involved in the up-regulation of SOD1 gene transcription, in particular to suggest innovative pharmaco-therapies using ASOs or small interference RNAs (siRNA) to counteract ALS.

Competing interests.

None of the manuscript's authors have relevant financial or nonfinancial relationships that might create a conflict of interest to disclose.

Acknowledgments.

This work was supported by Italian Ministry of Health (Grants Ricerca Corrente 2013–2015).

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Tables Legends.

Table 1. MS/MS analysis on *SOD1*-ARE and TFs complexes at basal condition (t0) and after 30 and 60 min of 1 mM H_2O_2 treatment.

TF	MW (kDa)	1mM H_2O_2			Coverage %
		tO	t30	t60	
Nrf2	**67.8**	✔		✔	24.11
NFkB p105	**105.3**	✔	✔	✔	34.3
AHR	**96.2**	✔	✔	✔	22.88
THRbeta	**52.8**	✔	✔	✔	44.27

Figure Legends.

Figure 1. PolII recruitment to *SOD1* promoter (A). ChIP analyses using antibody against RNA PolII was performed as explained in Materials and Methods. SH-SY5Y cells were treated with 1 mM H_2O_2 for 30, 45 and 60 min. The results have been normalized on the % occupancy of background (beads without antibody) and are expressed as mean ± SD. The image shows significantly increased recruitment of PolII association to the region encompassing the TSS (***p value < 0.001) after H_2O_2 treatment. The data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; n=3. **Schematic representation of *SOD1* gene** and the position of primers pairs used in qRT-PCR ChIP experiments for *SOD1* gene **(B)**.

Figure 2. ARE sequences in *SOD1* promoter (A). The table shows the ARE sequences in *SOD1* promoter, used to design EMSA and pull down probes, and the ARE sequences on the positive control gene *NQO1*. These sequences have been compared with Nrf2 consensus sequence derived from literature data (image below). **ARE transcription complex on *SOD1* promoter (B).** Nuclear extract from SH-SY5Y cells treated for 30, 60 and 120 min with 1 mM H_2O_2 were incubated with a DNA probe IRDye ®700, as described in Materials and Methods. The arrows indicate the transcriptional complexes formation. The image is representative of three independent experiments. The panel represents the average values of the 3rd shifted band. Results were expressed as mean ±

SEM; n=3. The data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; **p < 0.01, ***p < 0.001.

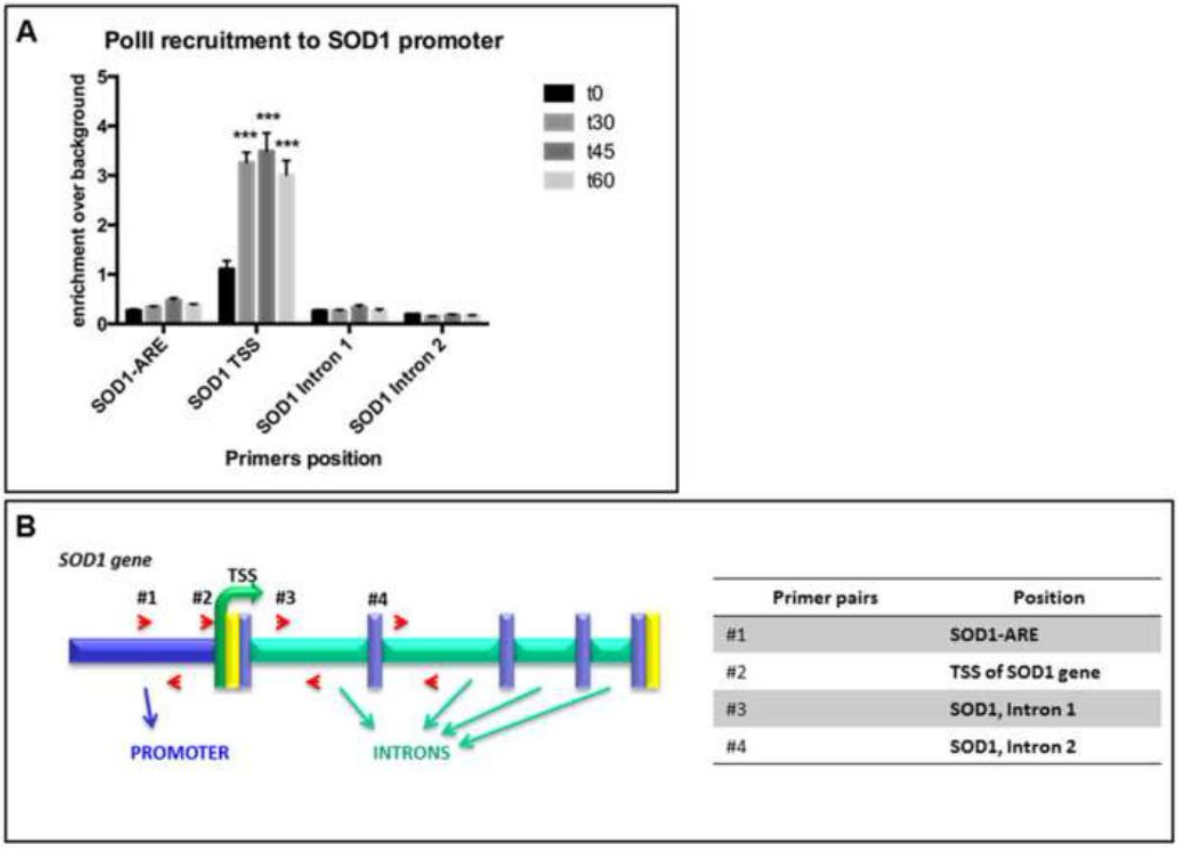
Figure 3. Nrf2 recruitment to *SOD1* promoter. ChIP analysis using antibody against Nrf2 was performed as explained in Materials and Methods. SH-SY5Y cells were treated with 1 mM H_2O_2 for 30, 45 and 60 min. The results have been normalized on the % occupancy of background (beads without antibody) and are expressed as means ± SD. The data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; n=3.

Figure 4. Nrf2 recruitment to *NQO1* promoter (A). Primer pairs were designed to flank the ARE elements and the TSS in *NQO1* promoter and in *HMOX1* exon 3 selected as positive and negative control respectively (right table). We showed significant Nrf2 association on *NQO1*-ARE sequences (**p value < 0.01) after H_2O_2 treatment. The data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; n=3 (A). **PolII and Nrf2 recruitment to *SOD1* promoter under tBHQ (B)**. ChIP analysis using antibodies against PolII and Nrf2 was performed as explained in Materials and Methods. SH-SY5Y cells were treated with 40μM tBHQ for 24 hrs. The results have been normalized on the % occupancy of background (beads without antibody) and are expressed as mean ± SD. The data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; n=3. We found increased PolII and Nrf2 association to the *NQO1* TSS region (***p value < 0.001) after tBHQ treatment.

Figure 5. Evaluation of Nrf2 TF activation in H_2O_2-treated cells. SH-SY5Y cells were treated with 1 mM H_2O_2 for 30 and 60 min and p-Nrf2 amount was quantified by WB (A). The p-Nrf2 expression was significantly increased in the nuclear and cytoplasmic fraction after the treatment both at t30 (*p < 0.05) and t60 (**p < 0.01) compared to t0. The amount of p-Nrf2 bound to Keap1 by IP followed by WB (B) as described in Materials and Methods. We reported a significant decline (**p < 0.01) at t60 compared to t30. Nrf2 in untreated cells was taken as 100%, data have been normalized on Keap1

immunoprecipitated levels and IgGs IP has been introduced to exclude non-specific binding. The image is representative of three independent experiments. Data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; **p < 0.01, n=3. **Nrf2 subcellular localization analysis in H_2O_2-treated cells.** Representative IF images of p-Nrf2 (green) intracellular localization in 60 min H_2O_2 -treated SH-SY5Y cells (H_2O_2 60 min). Scale bar: 15 µm. The IF analysis shows Nrf2 accumulation in the nuclear compartment of H_2O_2 treated cells.

Figure 6. Determination of *Nrf2* and *NQO1* mRNA levels by qRT-PCR in human neuroblastoma SH-SY5Y cells following treatments with 1 mM H_2O_2. mRNA expression in untreated SH-SY5Y cells was taken as 100%. The values obtained from total cellular mRNA have been normalized to the level of *GAPDH* mRNA and expressed as mean ± S.E.M. Data were analyzed by ANOVA, followed by Newman-Keuls Multiple Comparison Test; *p < 0.05, n=3.



Primer pairs	Position
#1	SOD1-ARE
#2	TSS of SOD1 gene
#3	SOD1, Intron 1
#4	SOD1, Intron 2

Figure 1

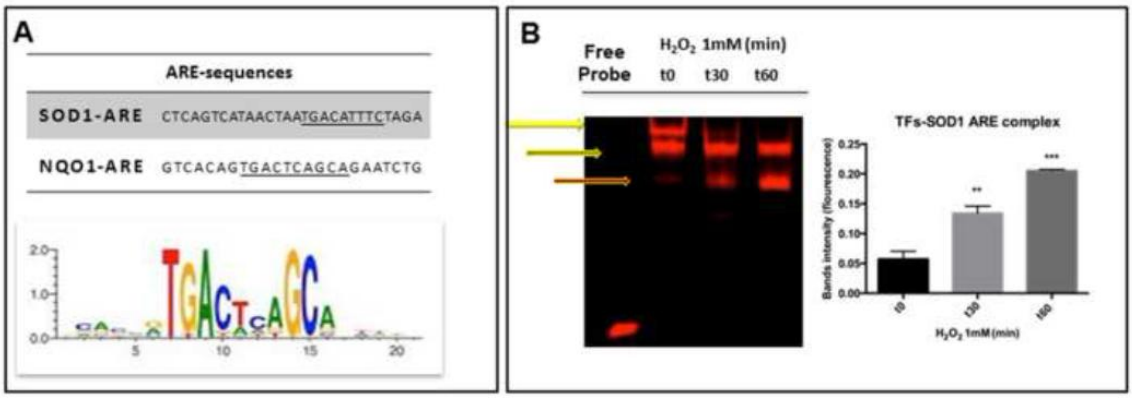


Figure 2

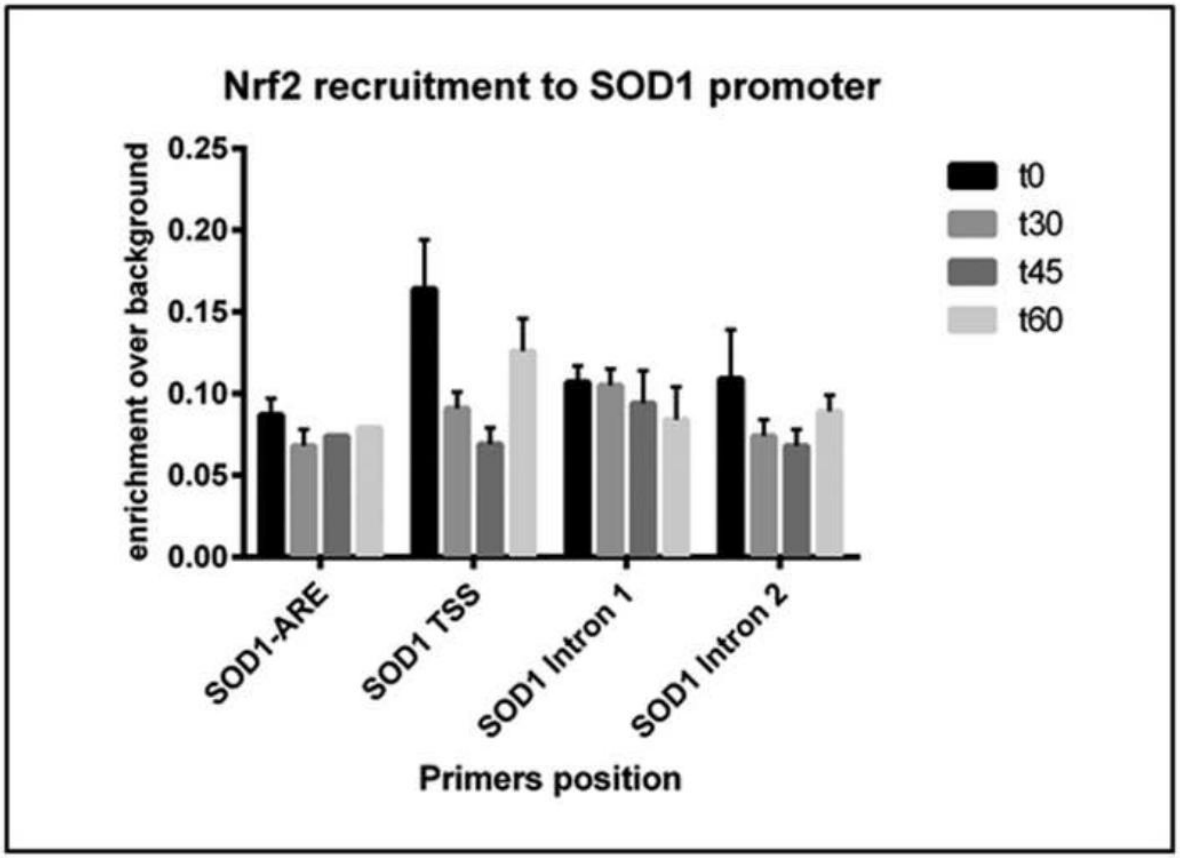


Figure 3

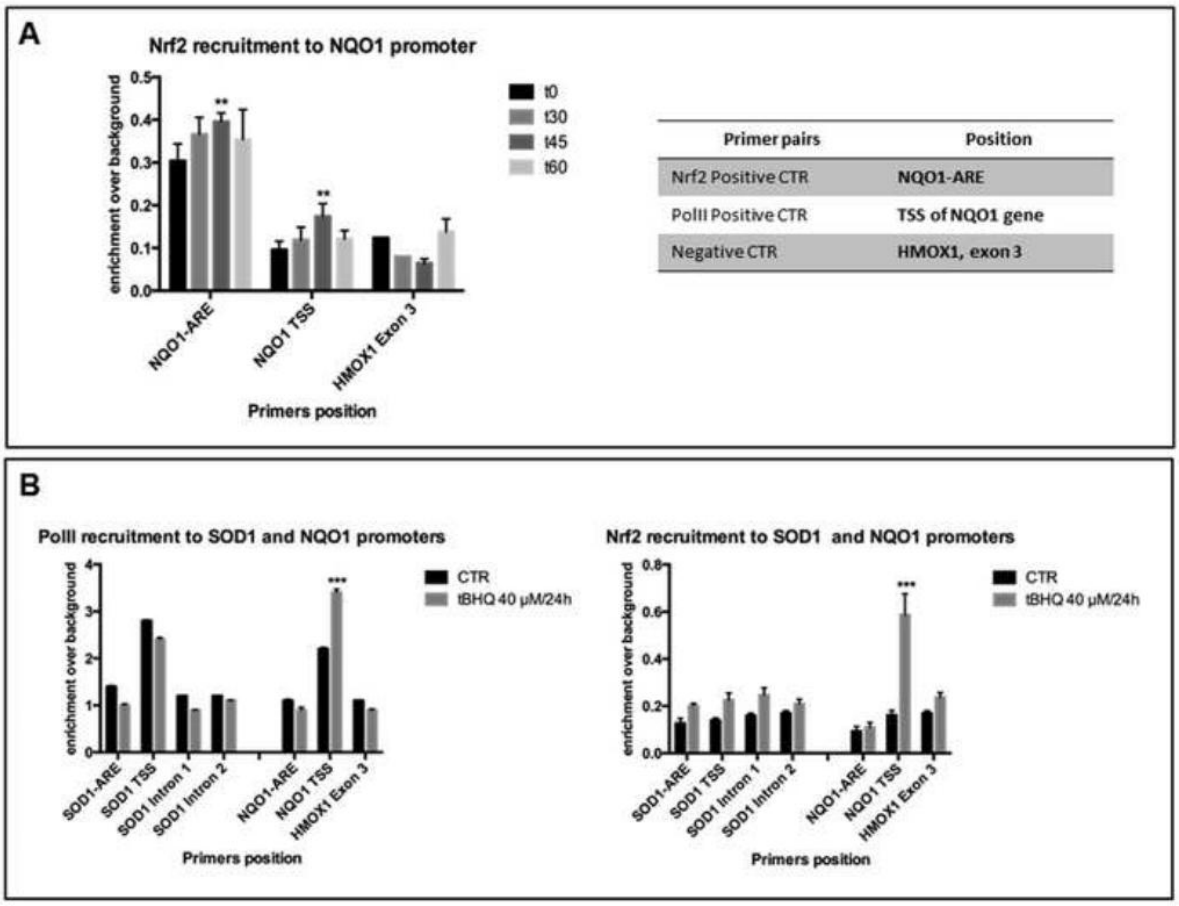


Figure 4

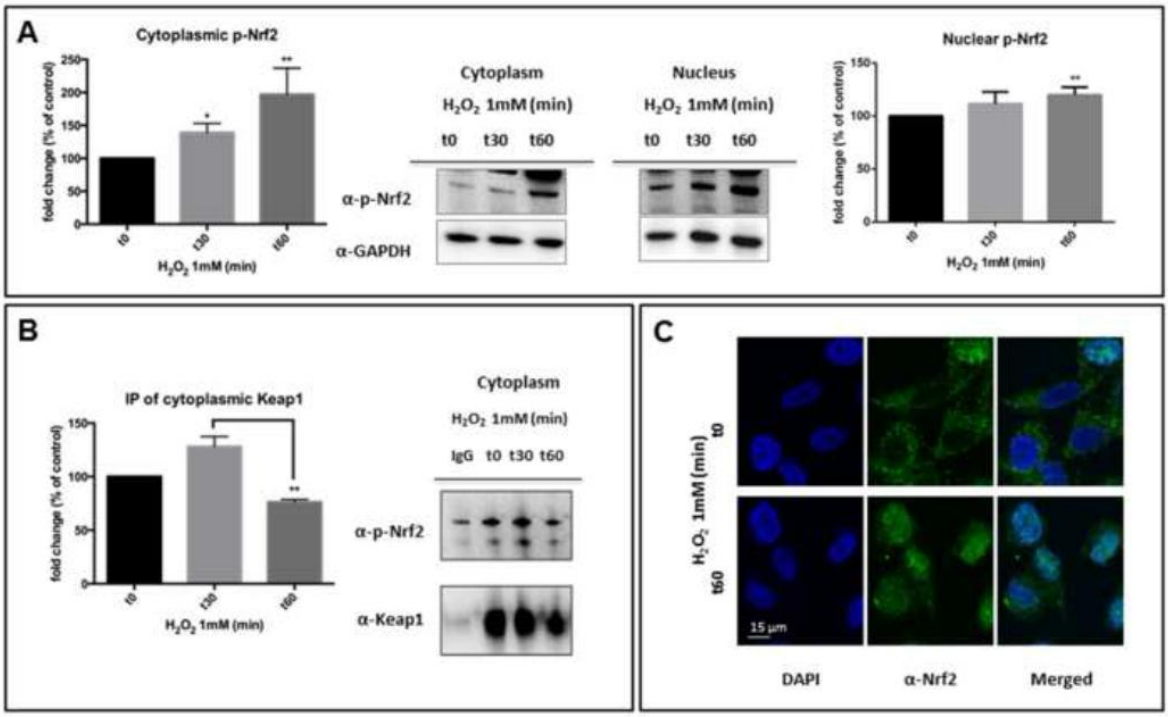


Figure 5

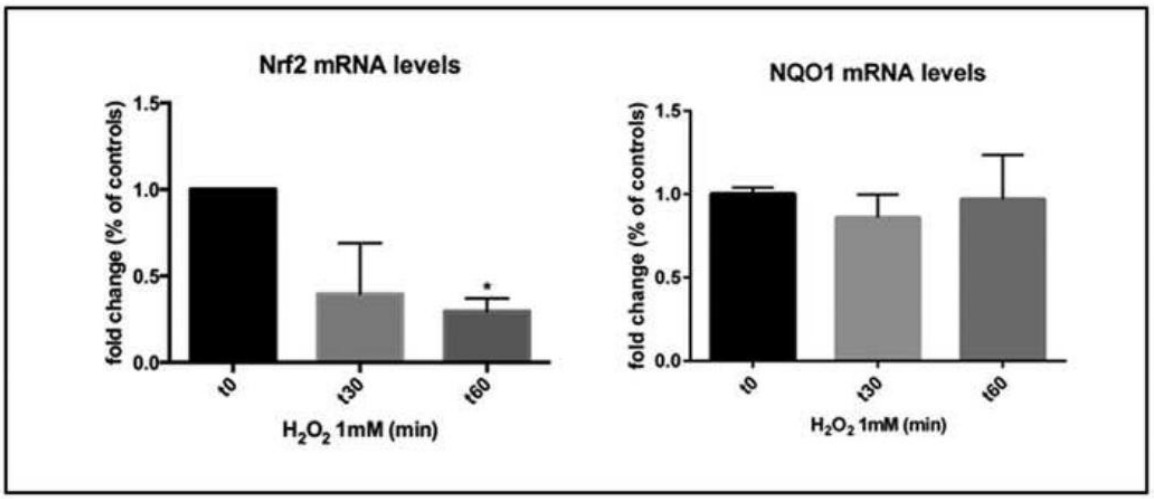


Figure 6

Highlights

- Increased SOD1 mRNA and protein levels are distinctive features of sporadic ALS;
- H_2O_2 activates *SOD1* gene transcription thanks to the formation of a multiproteins complex;
- we have no evidences that *SOD1* inducible expression is regulated by Nrf2;
- new insight into understanding the role of *SOD1* and Nrf2 in neurodegeneration;
- attempting to uncover biomarkers and therapeutic targets.