



## SOD1, more than just an antioxidant



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### ABSTRACT

During cellular respiration, radicals, such as superoxide, are produced, and in a large concentration, they may cause cell damage. To combat this threat, the cell employs the enzyme Cu/Zn Superoxide Dismutase (SOD1), which converts the radical superoxide into molecular oxygen and hydrogen peroxide, through redox reactions. Although this is its main function, recent studies have shown that the SOD1 has other functions that deviates from its original one including activation of nuclear gene transcription or as an RNA binding protein. This comprehensive review looks at the most important aspects of human SOD1 (hSOD1), including the structure, properties, and characteristics as well as transcriptional and post-translational modifications (PTM) that the enzyme can receive and their effects, and its many functions. We also discuss the strategies currently used to analyze it to better understand its participation in diseases linked to hSOD1 including Amyotrophic Lateral Sclerosis (ALS), cancer, and Parkinson.

### 1. Introduction

Reactive Oxygen Species (ROS) are a byproduct of the aerobic metabolism. To cope with this natural source of ROS, aerobes are equipped with a sophisticated antioxidant defense system which counteracts and controls ROS levels to maintain physiological homeostasis [1].

Superoxide dismutases (SOD) are a class of enzymes that catalyze the conversion of superoxide radicals to oxygen and hydrogen peroxide. SOD activity relies on a specific catalytic metal ion, which could be either manganese (MnSOD), iron (FeSOD), nickel (NiSOD), or copper (Cu/ZnSOD) [2]. These classes of the SOD molecule have evolved in various organisms; in eukaryotes, MnSOD and Cu/ZnSOD are the major isoforms [3–6].

All mammals (including humans) possess three isoforms of superoxide dismutase: Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3). Either the impairment of their antioxidant function or the toxic unknown gain of function have been associated with diseases, such as atherosclerosis, linked to MnSOD [7], and diabetic retinopathy, associated with serum extracellular Cu/Zn-SOD [8]. Down syndrome [9], Parkinson's disease [10], cancer [11], and Amyotrophic Lateral Sclerosis (ALS) [12] have been associated with SOD1 abnormalities. Recently, Park et al. discovered a severe and fatal syndrome caused by a homozygous truncating variant in human SOD1,

distinct from ALS, which completely abolishes enzyme activity [13], reinforcing the overwhelming importance of this enzyme to health. At one time, SOD1 was considered not essential and its silencing was proposed as a treatment option for neurodegenerative diseases [13]. However, studies with SOD1 knockout mice have shown that although these animals do not show any signs of neuromotor disease, they exhibit increased oxidative stress related to mitochondrial dysfunction, decreased lifespan, development of hepatocellular carcinoma, among other changes [13]. Therefore, these modifications must be taken into account by studies focused on silencing SOD1 as a possible treatment for ALS.

Human SOD1 (hSOD1) is responsible for regulating the superoxide levels arising from mitochondrial intermembrane space, cytosol, and peroxisome [14,15]. In addition to being an antioxidant enzyme, some new functions of hSOD1 protein have been reported, including activation of nuclear gene transcription following exposure to oxidative stress [16], regulation of RNA metabolism [17,18], and modulation of glucose sensing pathway to repress respiration [19]. Some regions of hSOD1 are likely to be intrinsically disordered [20]. Structural flexibility, due to the absence of a definite ordered structure, is considered a functional advantage for hSOD1 that might enable this protein to interact with different partners, for example other proteins, membranes, and nucleic acids, making hSOD1 a multifunctional protein.

In 1993, Rosen et al. identified SOD1 mutations related to fatal adult-

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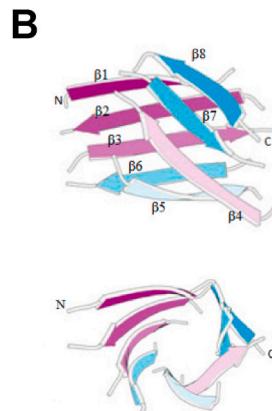
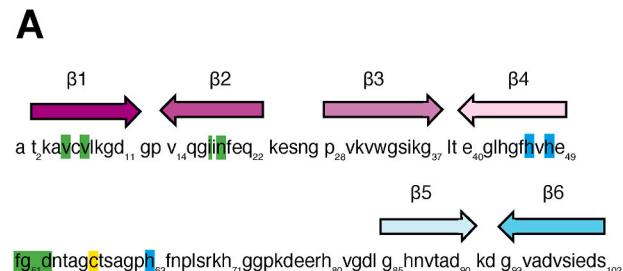
onset neurodegenerative cases of familial ALS (fALS), which indicated that hSOD1 is a contributor factor for aging and neurodegeneration [12]. Those authors also explored some interesting links and converging themes involving oxidative stress, fALS-linked hSOD1 mutants, and aggregates. More recently, some studies have provided insights into the role of hSOD1 in tumorigenesis. The functional relationship between hSOD1 and TORC1, the major mediator of nutrient signaling, suggested that regulation of hSOD1 is important for moderating oxidative stress and sustaining cancer cell survival under ischemic tumor microenvironment [21]. Consistently, inhibition of hSOD1 induced cell death in NSCLC (non-small cell lung cancer) cells, including those harboring KRAS mutations [22].

Modern analytical tools and diverse model organisms have been used to investigate cellular pathways that rely on this abundant and ubiquitous protein [6,23]. Advances in our knowledge of hSOD1 biochemistry as well as the mechanisms of hSOD1 regulation are critically analyzed in this review. Additionally, we discuss the recent advances in understanding the new hSOD1 functions in different subcellular compartments as well as the enormous relevance of altered hSOD1 for neurodegenerative diseases and cancer development.

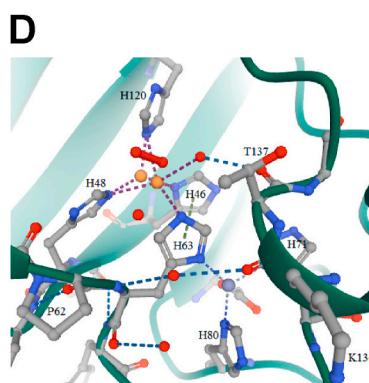
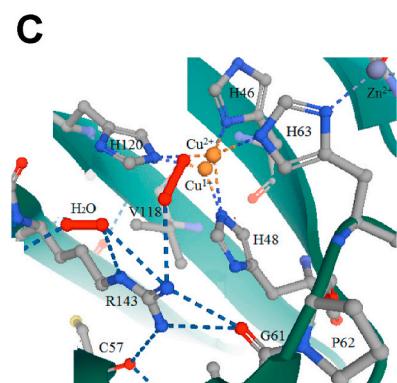
## 2. hSOD1 characteristics

### 2.1. Structure and stability

Human SOD1 is a homodimeric protein, 32 kDa, in which each monomer is formed by 153 amino acids (15.8 kDa), and its gene is localized on chromosome 21 (locus 21q22). This ubiquitously expressed protein represents between 1 and 2% of total proteins in cells [24]. It can be found in cytosol, nucleus, mitochondrial intermembrane space, and peroxisomes of human cells, according to its function and condition [20]. Studies have already proven that Cu/ZnSOD is a highly conserved protein among eukaryotic organisms, such as mammals, plants, and fungi [25].



**Fig. 1. Structure representation of human SOD1.** (A) Amino acid sequence of hSOD1 monomer. The residues in the buried region of β-strands were also identified. Highlighted in green, residues localized in the dimeric interface; in red and blue, residues related to the stability and activity of the catalytic core; in yellow, residues C57 and C146, which form the disulfide bond to stabilize the hSOD1 monomer. The arrows represent each β-strand and the residues that constitute them. (B) Structural representation of the eight β-strands of a hSOD1 monomer that acquire the Greek key β-barrel conformation. N represents the amino terminus and C represents the carboxyl terminus of the protein (Adapted from Wright et al., 2019[27]). (C and D) Structure representation (RCSB-PDB: 2C9V) of the coordination between Cu (orange balls) and the residues related to the stability and activity of the catalytic core (nitrogen, oxygen and sulfur atoms are blue, red and yellow, respectively). H<sub>2</sub>O molecules are represented as red sticks and Zn is represented by a light blue ball.



protonated, the binding to Zn is maintained. A second superoxide ion binds to the Arg143 residue and receives  $1e^-$  from the oxidation of  $Cu^{1+}$  to  $Cu^{2+}$  and  $2 H^+$ , forming  $H_2O_2$ . Once  $Cu^{2+}$  is regenerated, its binding is restored to the His63 residue, to be able to dismutate more superoxide ions [28].

hSOD1 is one of the most thermostable dimeric enzyme found in mesophilic organisms [26]. Biochemical and biophysical studies have tried to determine what is behind its stability even in unfavorable conditions, such as high temperatures, pH variations, and denaturing conditions found in the cytoplasm, a cellular location where most of the generated hSOD1 is located [24]. To reach its stable and active dimer form, hSOD1 undergoes 3 essential post-translational modifications (PTM): binding to Cu and Zn ions and forming the disulfide bond between Cys57 and Cys146 residues. Each hSOD1 monomer undergoes these modifications, and some authors believe that the dynamics between these modifications is what gives hSOD1 its stability [31]. These modifications provide the correct folding of the hSOD1 quaternary structure, as well as its stability [26]. Finally, the dimerization of hSOD1 occurs through hydrogen bonding between the dimer interface amino acid residues of the stabilized and active subunits (Fig. 2) [32].

This interaction has high resistance to dissociation even in unfavorable conditions, as already demonstrated by some authors working in denaturing conditions, such as 8–10 M urea solutions, 7 M guanidine, or 10% SDS [26]. In addition, hSOD1 only melts at temperatures above 90 °C [33].

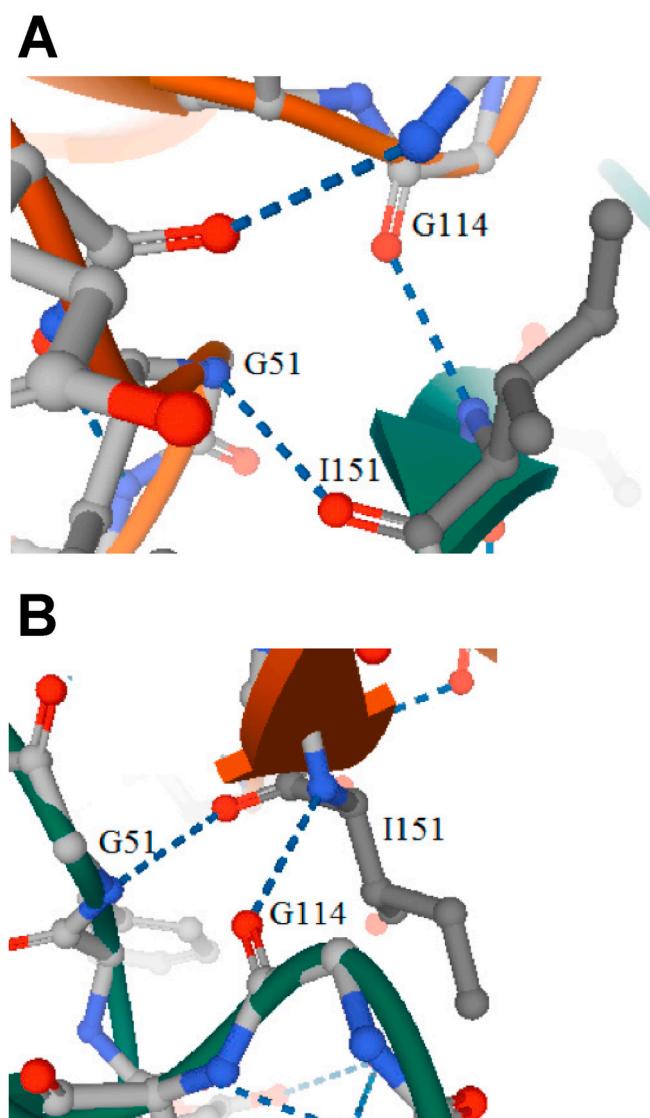
Initially it was believed that the structural stabilization of hSOD1 monomer was exclusively due to the formation of the disulfide bond between Cys57 and Cys146, which connects the loop IV to one of the  $\beta$ -strand [34]. This interaction, adjacent to the dimeric interface, favors the interaction of the two subunits, which leads to the formation of the hSOD1 dimer [26]. The formation of this intramolecular bond in hSOD1 monomers is a particular characteristic of this protein, since cytosolic proteins tend to not maintain this type of bond in the denaturing conditions of the cytoplasm [33].

However, further studies showed that the formation of the disulfide bond occurs after the acquisition of  $Cu^{2+}$  and  $Zn^{2+}$  ions [34]. NMR studies have found that the binding of the Zn ion helps in the structural organization of the non-matured hSOD1 monomer, showing that the coordination with this metal increases its structural stability [31,35]. Molecular dynamics studies indicate that the insertion of the Zn ion also stabilizes the dimeric form of hSOD1, reducing the structural flexibility of the components of the dimeric interface, which reduces variations in this region [31].

## 2.2. hSOD1 as an intrinsically disordered protein

Human SOD1 is part of the group of proteins with intrinsically disordered regions or intrinsically disordered proteins (IDPRs/IDPs), which are characterized by not having a defined secondary/tertiary structure under physiological conditions [36]; thus, proteins can change their structural conformation quickly [37]. Due to these characteristics, IDPRs/IDPs are able to bind to different molecular partners, resulting in several functions such as regulation, signaling, and control [20]. The degree of intrinsic protein disorder increases with the complexity of the organism, and more than half of the proteins of eukaryotic organisms are classified as IDPRs/IDPs [37].

It is no different with hSOD1. With the use of different algorithms (for example the PONDR family or some servers, such as IUPred web server), studies in the literature have already determined that approximately 40% of the hSOD1 residues present a disordered conformation [38]. In addition, Santamaría et al. demonstrated, using the ANCHOR algorithm, that human SOD1 has 5 disordered regions, which are 1–9; 32–38; 42–49; 98–119; and 144–153 [38]. The importance of these regions for the different functions that hSOD1 performs is also demonstrated by comparing these regions of human SOD1 with the SOD1 sequence of *Hyda attenuata*, which shows that they are conserved regions



**Fig. 2.** The dimer interface between two hSOD1 monomers. A and B illustrate the dimer interface, where each monomer is highlighted in green and orange (RCSB-PDB: 2C9V). The residues G51, G114, and I151 of each monomer interact through hydrogen bonding (dashed blue line) to form the stable and active hSOD1 dimer. Specifically, N-H of G51 and C=O of G114 of one monomer hydrogen bond to C=O and N-H of I151 of the other monomer, respectively.

and present the same degree of disorder [38].

Usually sites subject to post-translational modifications are found within these regions [37]. Analyzing some important hSOD1 PTM, modifications, such as phosphorylation of Ser98 (nuclear location of hSOD1) [16] and Thr2 (stabilization of the monomer/dimer balance of hSOD1) [39] and palmitoylation of Cys6 (maturation and localization of hSOD1) [40], are found within the regions considered to be disordered, indicating a possible importance between the presence of these disordered regions and the different functions that the hSOD1 protein exerts.

Recent studies have linked IDPs/IDPRs with diseases, such as neurodegenerative diseases and cancer. Studies with hSOD1 mutants related to the familial form of ALS found that some mutations can interfere with the degree of disorder in some regions of the protein [38]. For example, the L143F mutation decreases the level of disorder in the C-terminal region of hSOD1, which is related to the binding between hSOD1 and the G3BP1 protein, interfering in the dynamics of the appearance of stress granules [38]. Other mutations also decrease the

level of disorder in other regions of hSOD1, such as A4V, H46R, G85R, and G93A [38]; however, studies must be done to identify what these changes cause in hSOD1 functions that can lead to neurodegeneration.

### 3. Different roles of hSOD1

#### 3.1. The canonical role of hSOD1 as an antioxidant enzyme and its newly discovered functions

Slightly reactive and negatively charged, the superoxide radical is involved in several reactions that produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as  $\text{H}_2\text{O}_2$  and peroxynitrite ( $\text{ONOO}^-$ ) [41–43]. Although superoxide is not considered to be a strongly oxidizing agent, it is still potentially deleterious when its enzymatic removal by SOD-catalyzed dismutation reaction is insufficient.

Superoxide radicals have been shown to inactivate proteins, which contain [4Fe–4S] cluster by oxidizing one iron, causing its release from the cluster. In addition to causing protein inactivation, the iron release leads to further oxidative damage [44,45]. Cellular reductants keeps the free iron reduced, enabling its reaction with  $\text{H}_2\text{O}_2$ , via Fenton's chemistry, to yield hydroxyl radical, one of the most reactive oxygen species. The hydroxyl radical reacts indiscriminately, with very high rate constants, with all types of biomolecules, damaging these biomolecules [46].

Ubiquitously expressed and highly conserved throughout evolution, the metalloenzyme Cu,Zn-SOD1 is described as the major regulator of antioxidant response [1,46]. Human SOD1 constitutes a front-line defense mechanism against oxidative stress, catalyzing the disproportionation of superoxide into oxygen and hydrogen peroxide, which is further eliminated by catalase, glutathione peroxidases, and peroxiredoxins [46]. Additionally, the reaction catalyzed by hSOD1 is extremely efficient, occurring at the almost diffusion-limited rate of  $\sim 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , which is  $\sim 10^4$  times the rate constant for spontaneous dismutation [46]. From bacteria to humans, the loss of SOD activity is linked to elevated levels of oxidative damage, including membrane lipid peroxidation, protein carbonylation, and DNA breakage [6,47–50].

Largely cytosolic, hSOD1 is widely distributed throughout the cell, including lysosomes, the intermembrane space of mitochondria (IMS) and endoplasmic reticulum [51–54]. A fraction of the active hSOD1 is localized within the mitochondrial IMS, and this localization is greatly influenced by its cooper chaperone, CCS [15]. Sturtz et al. who studied yeast cells, found that when CCS synthesis was repressed, yeast Sod1 (ySod1) accumulated poorly in the mitochondria, and conversely, IMS ySod1 was very high when CCS was largely mitochondrial [15]. Thus, those authors concluded that the mitochondrial form of ySod1 is protective against oxidative damage, since yeast cells expressing high levels of IMS ySod1 exhibited prolonged survival during stationary phase [15]. Recently, Montllor-Albalate et al. demonstrated that only the IMS ySod1 (less than 1% of total Sod1) is necessary to protect from superoxide toxicity [55]. Thus, the much larger amounts of extra-mitochondrial ySod1 seem to play different roles, such as the regulation of Yck1, discussed in topic 3.4, corroborating the idea that SOD1 is more than just an antioxidant.

Similarly, hSOD1 can also be targeted to peroxisomes using its physiological interaction partner CCS as a shuttle [14], demonstrating that the SOD1/CCS co-import is a physiologically relevant piggyback import into mammalian peroxisomes [14]. Although ySod1 has also been identified in peroxisomes, mitochondria, and nucleus, neither CCS nor ySod1 (or hSOD1) contains typical address label sequences at N-terminal to mediate the localization in these organelles [14–16]. Although heterologous protein complexes between hSOD1 and CCS were proposed as a classic import mechanism more than a decade ago, the our understanding remains incomplete about how hSOD1 is directed to specific organelles and the reasons underlying this distinct hSOD1 localization.

hSOD1 is also found in the nucleus playing an important role as a transcription factor in response to oxidative stress [16,56]. Studies have clearly demonstrated that despite the antioxidant function, hSOD1 may play a greater role in cells than originally realized, acting as a key modulator of glucose signaling as well as an RNA binding protein [17–19]. The understanding of the recent advances of all the unconventional roles of hSOD1 and the identification of the critical cellular processes in both health and disease are discussed below.

#### 3.2. hSOD1 as a transcription factor in response to oxidative stress stimuli

Additional to its conventional role in superoxide radical scavenging, some new evidence indicates a function of hSOD1 in redox signaling for maintenance of genomic stability by altering subcellular localization [16,57]. In response to increased  $\text{H}_2\text{O}_2$  concentrations, ySod1 is phosphorylated at Ser59 and Ser98 by Dun1 via Mec1 cascade activation and rapidly relocates into the nucleus [16]. Mec1 is a DNA Damage Response (DDR) kinase. Chromatin immunoprecipitation demonstrates that, once inside the nucleus, ySod1 binds to DNA promoters to regulate the expression of genes involved in oxidative stress resistance, acting as a transcription factor [16]. Comparison of the expression profiles of WT and mutant sod1 revealed a hundred genes involved in cellular defense against ROS, replication stress, DNA damage repair, and Cu/Fe homeostasis, whose induction by  $\text{H}_2\text{O}_2$  was significantly attenuated by SOD1 deletion [16]. Peroxide also induces hSOD1 nuclear localization in human fibroblasts in an ATM DDR kinase-dependent manner, and Ser59 and Ser98 of hSOD1 are also found to be phosphorylated.

A recent study, conducted by our group, showed that during chronological aging, human SOD1 is localized in the nucleus and stimulates cellular defense mechanisms against oxidative stress by induction of genes involved in ROS resistance [58]. However, hSOD1 carrying the mutations (A4V, L38V, G93A, and G93C) of fALS formed cytoplasmic inclusions impairing its ROS-regulated shift to the nucleus, compromising the antioxidant cell response as well as cellular longevity [58,59]. In addition, we found that catalase (Ctt1) activity substantially increased in yeast cells expressing hSOD1WT after aging, compared to that cells expressing fALS-linked mutants ySod1. The nuclear hSOD1WT localization may induce genes involved in ROS resistance, such as CTT1 [58].

These observations indicate the nuclear function of hSOD1 as an efficient strategy to regulate gene expression in response to elevated ROS, preventing genomic damages, and possibly reversing ALS disease progression.

#### 3.3. hSOD1 action in the regulation of specific RNA stability

RNA dysregulation appears to be a major contributor to ALS pathogenesis, although the basic mechanism of its degenerative pathogenesis remains poorly understood [17]. Among the important, established causal ALS genes, SOD1, TDP43 (Transactive response DNA Binding protein 43 kDa), and FUS (Fused in Sarcoma) are deeply involved in aspects of RNA metabolism processes, such as mRNA transcription, alternative splicing, RNA transport, mRNA stabilization, and microRNA (miRNA) biogenesis [60]. In addition, RNA binding proteins (RBPs) play an important role in neurodegeneration. TDP43 is a highly conserved protein that contains two RNA Recognition Motifs (RRMs) and a C-terminal Glycine Rich Domain [61–64]. Similar to TDP43, FUS is an ubiquitously expressed nuclear protein which is also located in an aggregated state in the cytoplasm of ALS brain cells [65,66]. FUS is an RNA/DNA binding protein containing an N-terminal QGSY-rich domain, an RRM, and several RGG-repeat regions [64,67].

Unlike TDP43 and FUS, hSOD1 does not contain RRMs. However, despite the lack of RNA binding motifs, research has demonstrated a crucial role of mutant SOD1 in regulating RNA metabolism by binding mRNAs and playing a specific role in their stabilization [18,68–70]. Specifically, mutant hSOD1 binds the 3' UTR of the NFL (Neurofilament

Light Chain) mRNA as well as the mRNA sequence of *VEGF* (Vascular Endothelial Growth Factor) resulting in the formation of other ribonucleoproteins complexes, such as TIAR and HuR, into aggregates [17,71]. For *VEGF*, these specific interactions with mutant hSOD1 impair the HuR function and reduce the levels of *VEGF* mRNA, which are protective factors for neuron motors during stress responses [17,18,68].

Moreover, hSOD1 mutants (G37R and G93A) bind NFL mRNA and negatively affect its stability in motor neuron-like NSC34 cell lines [70]. Menzies et al. also described the decline of NFL mRNA levels in G93A transgenic mice and motor neurons derived from the spinal cord of ALS patients [70]. It has also been suggested that the mRNA NFL destabilization by mutant hSOD1 causes the neurofilament aggregation of motor neurons and neurite degeneration in IPSC-derived models of ALS [69]. Taken together, these studies pointed out a critical role of mutant hSOD1 in RNA dysregulation in ALS pathogenesis.

Mutant hSOD1 notably destabilizes NFL mRNA [72], while TDP-43 can stabilize NFL mRNA [71,73,74]. The hSOD1 interaction with TDP43 has been shown to modulate *NFL* 3' UTR mRNA stability [74]. *In vivo* studies also demonstrated that only TDP-43 and either mutant or wild-type SOD1 co-localize in ALS motor neurons, suggesting that both TDP43 and SOD1 may equally contribute to the regulation of specific RNA stability [74].

By using next-generation sequencing (NGS) technology to study RNA dysregulation in a cellular model of motor degeneration, Rotem et al. found that RNA expression differs between the somatic and axonal compartments of the neuron, for both mRNA and miRNA [75]. The analysis of two ALS-related mutations (hSOD1 G93A and TDP43 A315T) changed mRNA and miRNA expression profiles and subcellular localization within both the soma and the axons, showing that most genes altered in TDP43 A315T were not dysregulated in hSOD1 G93A, and vice versa. These findings provide a comprehensive subcellular evaluation of the motor neuron transcriptome, both in healthy and diseased neurons [17,75].

### 3.4. *SOD1*, a key component of nutrient signaling

Both hSOD1 and hSOD2 protect cells from the superoxide production during mitochondrial respiration, acting as the first line of defense against oxidative damage by providing an efficient control of cellular ROS level. Moreover, hSOD1 has been indicated as a regulatory protein for diverse cellular processes such as signaling and respiration [21,76].

Energy metabolism is a process by which the biochemical energy derived from nutrients, in the form of ATP, is used to fuel biosynthesis and cellular growth. The two central pathways used by eukaryotes to produce energy are glycolysis in the cytosol and oxidative phosphorylation (OXYPHOS) in the mitochondria. Environmental nutrients determine which energetic pathway is used for ATP production and directly impacts ROS production [21].

In cancer cells, the metabolic shift from oxidative phosphorylation in the mitochondria to glycolysis in the cytosol was first described 90 years ago by Otto Warburg [77], and this phenomenon was called the “Warburg effect” [78–80]. Like cancer cells, the budding yeast *Saccharomyces cerevisiae* predominantly uses glycolysis when glucose is available even in the presence of oxygen, a phenomenon called “aerobic glycolysis” or the “Crabtree effect” [81]. When only a non-fermentable carbon source is available, yeast switch to OXYPHOS [81].

Two different complexes are formed by mechanistic target of rapamycin (mTOR): mTORC1 and mTORC2. As a nutrient sensor, mTORC1 is an important regulator of cell growth and metabolism [82–84]. In both yeast and human cells, the target of rapamycin 1 (mTOR) complex regulates Sod1 activity in response to the availability of nutrients, which moderates ROS level and prevents oxidative DNA damage [21]. Thus, hSOD1 activation may increase cancer cell survival and tumor formation [21].

In a very elegant study, Reddi and Culotta reported that ySod1 can transmit signals from oxygen and glucose to repress respiration, by using

a yeast model system [19]. The proposed mechanism involves ySod1 stabilization of casein kinase 1-gamma (CK1 $\gamma$ ) homologues required for nutrient sensing, Yck1 and Yck2 [19]. Yeast Sod1 physically binds to a C-terminal region in Yck1 preventing their degradation via its Sod enzymatic reaction, resulting in Yck1 stabilization [19]. Then, through inhibition of Mth 1 and Rgt1 [85], the hexose transporter genes (HXT) are induced, which favors aerobic fermentation [19]. In the absence of ySod1, Yck1 and Yck2 are degraded, leading to aerobic respiration [19, 86]. Interestingly, those authors also observed that the hSOD1 stabilization of CK1 $\gamma$  could be extended to other eukaryotes, such as humans and other mammals [19]. By using the human cell line HEK293 treated with a hSOD1 inhibitor, the intracellular copper chelator tetra-thiomolybdate (TTM), they demonstrated a drastic decrease in levels of human CK1 $\gamma$ , corroborating the yeast expression studies [19]. Conversely, enzymatically active hSOD1 promoted CK1 $\gamma$  expression in human cell line, suggesting a conserved role for hSOD1 in stabilizing CK1 $\gamma$  isoforms and regulating fermentative metabolism [19].

CK1 isoforms have long been associated with cancer progression in different types of tumors [87]. Since the relation between CK1 $\gamma$  and the higher expression of glucose transporters is reported, we can hypothesize whether the hSOD1 may act to facilitate aerobic glycolysis in cancer cells.

Glucose repression of O<sub>2</sub> consumption appeared defective in yeast cells lacking the ySod1 protein (*sod1* $\Delta$ ) [88]. Based on all this evidence, our group investigated the role of hSOD1 in the aerobic glycolysis process. Preliminary results (not yet published) suggest that yeast *sod1* $\Delta$  cells expressing the hSOD1 ( $\Delta$ *sod1*hSOD1) exhibited a higher specific growth rate, a reduced O<sub>2</sub> consumption, and an elevated glucose consumption, compared to *sod1* $\Delta$  cells. We propose that the presence of hSOD1 could restore the glucose repression capacity that had been defective in the absence of the endogenous ySod1. These novel results suggest a conserved role of hSOD1 in metabolic signaling pathways that work in concert to repress respiration in yeast cells.

## 4. SOD1 regulation

In addition to understand its functions in cells, it is important to know how hSOD1 is regulated. In the last two decades, a few works have focused on the transcriptional regulation and post-translational modifications, which affect the level, structure, stability, localization, and functions of SOD1, as described below.

### 4.1. Transcriptional regulation

The mechanisms, especially regulation of genetic flow, behind cellular responses to different stimuli (intra and extracellular) are important to understand the levels of gene expression are modulated [89,90]. Studies have elucidated the importance of transcriptional and post-transcriptional modifications in cell response mechanisms. In this context, regulation of the mRNA is consider one of the most important steps for genetic regulation and better cellular response [91].

Oxidative stress is one of the most studied cellular conditions, mainly due to its relation to aging associated diseases, including ALS. When the cells are under oxidative conditions, the antioxidant defenses are activated. The levels of expression of the genes related to the antioxidant defense are regulated by transcriptional and posttranscriptional mechanisms [18]. As already mentioned in this review, hSOD1 is a protein for the cellular antioxidant defense. Some studies have focused on better understanding the structure, mechanism, and regulation of hSOD1 mRNA levels under normal and oxidative conditions [89].

The polypeptide of the hSOD1 monomer is encoded by the *SOD1* gene, with 5 exons interrupted by 4 introns in its coding region. The promoter of the *SOD1* gene, which regulates its expression at basal levels, has important regulatory factors such as TATA-binding protein, CCAAT/Enhancing protein (C/EBP1) and specificity protein 1, which binds with specific transcription factors such as Sp1, Egr 1, Nrf2, and NF-

$\kappa$ B [54,89,91]. In addition, it is believed that the expression of SOD1 could be regulated by the partial overlapping of the C/EBP and specificity protein binding that activates a coordinated response to different molecular signals in the cell. However, more studies must be done to better understand this type of regulation [54].

Specificity protein 1, Sp1, when overexpressed, regulates hSOD1 binding in the promoter site, and enhances its basal promoter activity. AP1 (Activating Protein 1) indirectly regulates hSOD1, repressing its transcription by kidnapping Sp1. Studies with HeLa cells under oxidative stress showed that the hSOD1 mRNA levels increased after treatment with phorbol-12-myristate-13-acetate (PMA), which induces Egr-1 (Early growth Response -1) expression, indicating the relation between this transcriptional factor and SOD1 expression [91].

Nrf2 (Nuclear Factor E2-Related Factor 2) and NF- $\kappa$ B (Nuclear Factor-KappaB) are transcriptional factors that induce hSOD1 transcription under oxidative stress [89]. Nrf2 is known as a key regulator of the antioxidant mechanism of human cells. Once it is activated by stressors inducers, Nrf2 can bind to *cis*-acting antioxidant/electrophile response elements (ARE/EpRE) present in the gene of some antioxidant proteins, among them hSOD1, to up regulate its transcriptions [91,92]. NF- $\kappa$ B was one of the first studied transcriptional factors related to antioxidant defenses. Several studies found that NF- $\kappa$ B is activated by H<sub>2</sub>O<sub>2</sub> in different cell types, leading to an up regulation of hSOD1 mRNA levels [89,91].

With the increasing studies focused on hSOD1 mRNA, the idea of it as a “housekeeping gene” has lost strength, because research identified that the amount of hSOD1 mRNA and protein are the result of a complex pathway regulated by different steps and elements, which can be negative and positive, at the same time [89]. Although no RNA-binding motifs have been identified [17], the hSOD1 mRNA structure presents in the 3'UTR portion some “adenine/uracil-rich stretches”, which suggests that the expression of hSOD1 occurs quickly in response to any change in the cell [89]. In addition, two different species of hSOD1 mRNA with two different lengths of the 3'\_UTR portion are involved in the regulation of mRNA metabolism and stability [91].

#### 4.1.1. Transcriptional regulation of yeast *Sod1*

The yeast *Saccharomyces cerevisiae* has been widely used as a study model to better understand metabolic bases, among them biochemical process conserved in cells of superior eukaryotes [93,94]. Its ability to grow in anaerobic and aerobic conditions helps make *S. cerevisiae* an advantageous model for studying the function of proteins that may be related to some human diseases, including cancer and neurodegenerative disorders. In anaerobic conditions (and high glucose levels),

*S. cerevisiae* can grow via fermentative metabolism (also known as catabolic repression, similar to the Warburg effect observed in tumor cells), a condition in which ROS level is low (without any type of stress). While, in aerobic conditions (respiratory metabolism), the level of ROS is high [95], which can lead to oxidative stress, observed in diseases related to cell aging, for example. Furthermore, its genome has been sequenced and characterized (only less than 1% remains uncharacterized), and 30% of *S. cerevisiae* genes have homologues in human genes, and 25% of human genes related to neurodegenerative diseases have homologues in yeast, among them hSOD1 (Fig. 3) [95]. Based on this, our group and others have used this model to determine, for example, the activity of hSOD1WT and mutant hSOD1, expressed in yeast cells without ySod1 endogenous (*sod1* $\Delta$ ), under different stress conditions, such as cell aging and oxidative stress induction [58,96]. Using *S. cerevisiae* cells as a model, several studies have shown the regulation of ySod1 expression by transcription factors in different stress condition, which enhance intracellular ROS levels, such as oxidative stress, thermal stress [97], and intracellular copper levels [98].

An important transcription factor that regulates the expression of the yeast *SOD1* gene is Msn2/Msn4. This is one of the main regulators of response to environmental stress by yeast cells [99] and are related to recovery from a stress condition, which may be oxidative stress, temperature shift, among others [100]. Studies of the ySod1 promoter region have already shown that close to this region, yeast cells have a STRE (stress response element) sequence [101]. Under stress conditions, active Msn2/Msn4 recognizes this sequence and induces activation of the *Sod1* promoter [102].

While MSn2/Msn4 has been more related to activation of recovery mechanisms against stress condition, Yap1 is a specific transcription factor that activates response mechanisms to adapt the cells under oxidative stress [103]. Yap1 is activated in different conditions, such as the presence of metals that generate an accumulation of ROS [103], oxidative stress generated by an increase in the concentration of ethanol, or induction by hydrogen peroxide treatment [104]. The increase in the intracellular concentration of ROS, activates Yap1, which is displaced from the cytoplasm to the nucleus, where it recognizes the antioxidant response sequence (ARE), activating the mechanisms of responses to oxidative stress, among them expression of the yeast *SOD1* gene, which has in its promoter region the ARE sequence [100,104].

In addition to the main transcription factors for ySod1 mentioned above, other factors regulate the expression of this protein in response to different cellular conditions, for example, intracellular Cu accumulation. Despite being an essential metal, in high concentrations, Cu can play a toxic role for cells [98]. Within its promoter region ySod1 has a



**Fig. 3.** Sequence alignment of human and yeast *SOD1*. Highlighted in pink are some conserved residues related to i) SOD1 catalysis role (H46, H48, H63, H120, and R143 exert a direct effect, while D83, D124, K136, and T137 indirectly affect catalysis); ii) SOD1 stabilization (C57 and C146, involved with intramolecular disulfide bond; and iii) SOD1 posttranslational modifications (Ser59 and Ser98 phosphorylation, which target SOD1 to nucleus; K136 ubiquitination). Highlighted in blue are T39 and S38 of human and yeast SOD1, respectively, which are not conserved; however, when phosphorylated they play the same regulatory role in SOD1. G51 and G114, required for dimer interface interaction, are conserved; the position 151, which also participates of dimer stabilization, is occupied by a leucine instead of an isoleucine in yeast Sod1. The fully conserved residues are indicated with an asterisk (\*). The colon (:) indicates conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix). A point (.) indicates conservation between groups of weakly similar properties (scoring = < 0.5 in the Gonnet PAM 250 matrix).

binding site for the Ace1 transcription factor, which is a member of the group of transcription factors that have a copper-fist cysteine DNA-binding domain [98,105]. Studies using strains deficient in Ace1 found that even under conditions with an increase in the concentration of this metal, the expression of *ySod1* mRNA does not increase, proving its importance as an activator of this protein under toxic concentrations of Cu [105]. Under conditions of low Cu concentration, Sod1 is also regulated by the transcription factor Mac1, which is activated by the release of Cu ions from its cysteines in response to a drop in the intracellular concentration of this metal [106].

#### 4.2. Post-translational modifications

Post-translational modifications (PTMs) are chemical modifications that regulate some characteristics of the proteins, such as structure, localization, functions, interactions, and turnovers [107]. These modifications can also play important roles in different biological pathways that are vital to maintain the correct cellular homeostatic balance [108]. However, not all PTMs are beneficial for cells or proteins. Some post-translational modifications can modify the protein structure, leading to wrong folding or even misfolding, believed to be the first step towards protein aggregation, which can be the trigger for some neurodegenerative disease, including ALS [109].

After hSOD1 transcription, hSOD1 is found in the cell in apo monomeric form, and to reach its active form, it must undergo some post-translational modifications. The structure and enzymatic function of hSOD1 is stabilized and activated primarily by these essential and well known PTMs: bind to Zn<sup>2+</sup> for structure stability; acquisition of Cu<sup>2+</sup> for enzymatic activity; intramolecular disulfide bond between Cys57 and Cys146; and homodimerization, by hydrogen bonding on the dimeric interface [110,27]. The Cu acquisition occurs by interaction between SOD1 monomer and the chaperone CCS [110].

Besides these PTMs, with the development of new techniques, especially Mass spectrometry, more posttranslational modifications in hSOD1 have been detected, such as phosphorylation, glycation, palmitoylation, acetylation [108], and more studies have focused on what chemical and biophysical properties they modify [27].

##### 4.2.1. Acylation: acetylation and succinylation

Acylation is a posttranslational reversible modification of lysine residues, which can be classified according to the size of the carbon chain: acetylation, for the addition of one carbon atom (reaction with acetyl CoA), or succinylation to add 2 carbon atoms (reaction with succinyl CoA). The lysine acylation of hSOD1 regulates its enzyme activity by modifying the surface charge, ranging from +1 to 0 (acetylation) or from 0 to -1 (succinylation) [111].

One of the most common posttranslational modification is lysine acetylation, which regulates activity and signaling of proteins on the cytosol, including hSOD1 [27]. Proteomics studies have shown that hSOD1 can be acetylated at Lys70. Lys70 is on the surface of zinc-biding loop, which is solvent exposed [27]. Acetylation of this residue harms the interaction of CCS with hSOD1, leading to a decrease in hSOD1 enzymatic activity [108] and the loss of hSOD1 homodimer formation [111]. SOD1 is also acetylated at Lys122, which inhibits the ability of hSOD1 to suppress respiration. Since, in yeast, *ySod1* interacts with Ck1γ to suppress respiration, one hypothesis is that Lys122 acylation might disrupt the SOD1-Ck1γ interaction [19,111]. However, *S. cerevisiae* has a glycine instead of lysine at position 122. Lys136 is another lysine that could modulate SOD1-casein kinase interaction, which is acetylated and conserved between yeast and human [112].

With the development of techniques to detect posttranslational modifications involving mass spectrometry, 2,565 susceptible sites of succinylation have been detected in 779 proteins in mammalian cells, including hSOD1. Lys122 from hSOD1 is a conserved residue in all vertebrates and can be either succinylated or acetylated [113]. Studies have not demonstrated that an enzyme that is responsible for the

succinylation of Lys122; thus, it is believed that this modification occurs non-enzymatically through the reaction between this residue and the substrate succinyl CoA [114]. Lys122 is found in the electrostatic loop, close to the His120 and Asp124 residues, which are responsible for the interaction between the cofactors Cu and Zn and hSOD1, regulating both hSOD1 activity and folding [113,115]. Lin et al. using a K122E mutant (mimics a SOD1 which is always succinylated), found a 45% drop in hSOD1 activity compared to hSOD1WT [113]. Immunoprecipitation and western blotting experiments evidenced that hSOD1 binds to SIRT5 (deacetylase of the sirtuin Family deacylase), which desuccinylates hSOD1, recovering its enzymatic activity, and in turn, decreasing ROS levels [113]. These authors also showed that the coexpression of SIRT5 and hSOD1 decreases ROS levels by 43% [113].

The study of the relationship between SIRT5 and the level of succinylation of hSOD1 has drawn attention mainly to the possible role of this protein in the regulation of cellular respiration. Studies suggest that hSOD1, once succinylated, breaks its interaction with another protein related to respiratory repression (which may be Ck1γ), so that in this condition the cells start to grow in a respiratory metabolism [115]. Understanding this regulatory mechanism is very interesting for the study of tumor cell metabolism, since they grow under the Warburg Effect (non-respiratory metabolism) and have a high level of hSOD1 expression. Studies by Lin et al. indicated that the regulation of hSOD1 by SIRT5 is extremely important for the growth of lung tumor cells [113], reinforcing the importance of understanding this relationship between SIRT5, hSOD1, Lys122, and regulation of cell growth.

##### 4.2.2. Palmitoylation

Palmitoylation, also known as S-acylation, is a reversible modification that covalently binds a fatty acid (in this case, palmitate) to cysteine residues [110]. It is related to the regulation of protein trafficking, localization in the membrane, stability, and interaction between proteins [110]. hSOD1 has 4 cysteine residues: Cys6, Cys57, Cys111, and Cys146. Residues Cys57 and Cys146 form a stable intramolecular disulfide bond and thus cannot be palmitoylated [40]. Antione et al. used hSOD1WT and mutant hSOD1 (A4V, G93A and G85R) expressing mutations in Cys6 and/or Cys111, which abolished the capacity of these residues to be palmitoylated, to identify that the Cys6 is the main residue palmitoylated, while Cys111 is also palmitoylated, but to a lesser extent [40].

As a protein originally from the cytoplasm, hSOD1 is palmitoylated in Cys6 and directed to the membrane [40], where it will then interact with the CCS chaperone and pass through the other stages to acquire the metal ions of Cu and Zn and form the dimer [110]. The direct relationship found between the amount of palmitoylated hSOD1 and its presence in the membrane confirms the role of this modification in directing cytoplasmic hSOD1 to the membrane [40]. hSOD1 palmitoylation levels are higher in the early stages of maturation, when the disulfide bond is not yet formed [110], suggesting its importance in the formation process of this intramolecular disulfide bond and the maturation of the hSOD1 dimer.

Although it is not fully known how palmitoylation can affect hSOD1 (in a positive or negative way), studies have already evidenced its relationship to both localization and enzymatic activity. Working with HEK 293 cells expressing hSOD1 WT carrying the C6S mutation, which abolished the ability of hSOD1 to be palmitoylated, Marin et al. found decreased enzyme activity, without altering the expression of this protein [116]. Other studies using hSOD1 with a Cys6 mutation unable to be palmitoylated have shown a decrease in enzyme activity, *in vivo* and *in vitro* [40], demonstrating that this modification plays a regulatory role in the enzymatic function of hSOD1.

Marin et al. also found that the ability of SOD1 to dislocate to the nucleus was disrupted with the abolition of palmitoylation in the C6S mutant [116]. These results suggest that hSOD1 must go through the process of palmitoylation to be able to move from the cytoplasm to the nucleus, where it can act as a transcription factor for the antioxidant

protection of cells. As hSOD1 is mainly palmitoylated in its apo form, with reduced intermolecular disulfide bond [110], and that it is phosphorylated to be transported to the nucleus, both modifications probably work together to determinate hSOD1 nuclear localization. Studies focused on this relationship would be interesting to prove this simultaneous role of both modifications. Mass spectrometry studies to measure the phosphorylation level of Ser59 and Ser98 in a hSOD1WT without the ability to be palmitoylated (C6S or C6A) could shed light on this possible relationship.

Antinone et al. showed, using hSOD1 mutant cells (A4V, G93A, and G85R) expressing the C6S and/or C111S mutations, that these mutants are also palmitoylated in the Cys6 residue and to a lesser extent in the Cys111 residue. This study also demonstrated that hSOD1 mutants are more palmitoylated than hSOD1 WT [40]. Some mutations in hSOD1, such as A4V, alter the conformation of hSOD1, which leads to an exposure of hydrophobic residues and cysteines, among them Cys6 [117]. Cys6 is normally buried in the structure of hSOD1WT [116]. This difference suggests that the Cys6 residue in hSOD1mut, as it is more exposed, may be more palmitoylated, justifying the results observed by Antinone et al. [40]. Thus, palmitoylation can probably stabilize the monomers of hSOD1 mutants, which are considered the initial step for the formation of toxic clusters observed in cell models of fALS [40].

#### 4.2.3. Phosphorylation

One of the PTMs of hSOD1 most studied in the last decade is phosphorylation. Human SOD1 has 12 sites liable to phosphorylation, and they have already been related to nuclear localization, as well as stability of the monomer/dimer balance and regulation of enzymatic activity [111]. The phosphorylation of serines 59 and 98 are related to nuclear localization of hSOD1 under oxidative conditions. Tsang et al. using a yeast model under oxidative stress, demonstrated that mutations in both serines lead to a loss of the ability of hSOD1 to relocate to the nucleus. The nuclear localization of hSOD1 is important so this protein can act as a transcription factor and activate other antioxidant mechanisms. In yeast, the process of phosphorylation of ySod1 occurs by the protein Dun1, but in humans this process is still unclear [16].

Threonine 2 is a hSOD1 phosphorylation site related to stabilization of the dimer/monomer balance. Fay et al. showed that the phosphorylation of Thr2 can stabilize the dimer of hSOD1WT and the hSOD1A4 V mutant by decreasing the concentration of monomers and consequently of inclusions observed in some mutants related to fALS [39]. Nevertheless, how the regulation and stabilization through phosphorylation of Thr2 occurs has not been elucidated.

Recently, Tsang et al. [21] demonstrated that the enzymatic activity of hSOD1 can be regulated by the mTORC1/TORC1 complex, according to the nutritional availability of the cellular environment. In yeast cells and cell models of different types of cancer, both yeast Sod1 and human SOD1 are phosphorylated by TORC1 at Ser38 and Thr39, respectively, by a nutritional signal. Under high nutritional concentration, mTORC1 is activated and phosphorylates hSOD1 at these sites, inhibiting the enzymatic activity of this protein. This is because although hSOD1 has a negatively charged surface, this protein has a positively charged tunnel through which the superoxide radicals flow until they reach the catalytic site. However, Tsang et al. showed that both Ser38 and Thr39 are at the entrance to this tunnel, and once phosphorylated, the region becomes negatively charged, not allowing the superoxide flow to reach the catalytic site, decreasing the enzymatic activity of hSOD1. Under starvation, mTORC1 is inhibited and does not phosphorylate hSOD1, which in turn allows the superoxide flow to reach its catalytic site. These results are of great interest, mainly for a better understanding of how tumor cells manage to survive even in conditions with low concentration of nutrients, and in the face of oxidative stress, since ROS concentrations in tumor cells remain high, as well as the expression of hSOD1 [21].

#### 4.2.4. Oxidative modifications

Another type of posttranslational modification occurs when cells are

subjected to high levels of oxidative stress, leading to oxidative damage. hSOD1 is one of the proteins with the highest propensity to suffer oxidative damage, due to its role in the dismutation of superoxide ion and the consequent generation of hydrogen peroxide (a type of ROS) [118], beyond its localization in animal cells (motor neurons are more exposed to oxidative stress) [119] and for being a metalloprotein [120]. One of the main pathways for protein oxidation is through the introduction of a carbonyl group in the histidine, lysine, arginine, threonine, and proline residues, in addition to the oxidation of cysteine residues [121]. Examples of oxidative damage suffered by hSOD1 are protein carbonylation and oxidation of cysteines. Depending on the oxidized residue, this can lead to a destabilization of the quaternary structure of hSOD1 and/or loss of its enzymatic activity [122].

Residues related to the stability and activity of the hSOD1 catalytic site, such as His46, His48, His63, His71, His80, His120, and Arg143 (Fig. 1C and D), as well as residues involved in attracting superoxide radicals to the catalytic site, such as Lys136 and Thr137, have a high propensity to undergo oxidative damage by the peroxide generated during the dismutation of superoxide radicals. Once released, H<sub>2</sub>O<sub>2</sub> can react slowly with hSOD1, modifying these residues near the catalytic site, which can lead to the disruption of the Cu and Zn bond and/or loss of catalytic activity, resulting in the inactivation of this enzyme [28].

Oxidation of one or more Thr2, Lys3, Cys6, Lys9, Thr54, Pro 62, Cys111, Arg115, and Thr116 residues, which are part of the hSOD1 dimeric interface, changes the local hydrophobicity, interfering in the correct folding of the quaternary structure of hSOD1 [118]. For example, the carbonylation of residues Lys3, Lys9, and Arg115 destabilizes both the dimeric and monomeric forms of hSOD1. Carbonylation of Thr54 and oxidation of Cys111 destabilize only the monomeric form of the protein. Oxidation of Cys6, which initially is buried in the structure of hSOD1, exposes this residue, leading to a destabilization of the β-sheet structure of hSOD1 [118].

Protein carbonylation itself is an intensively studied irreversible oxidative damage. hSOD1 mutants, characteristic of fALS, such as the G93A, A4V, and L38V mutation, present higher levels of carbonylation than SOD1WT, in models of neurogliom. Moreover, the level of oxidative stress in these mutants is higher [59].

#### 4.2.5. Oxidative modifications in Cys111

Post-translational modifications in Cys111 of hSOD1 is one of the most studied because its residue is located close to the dimeric interface. Cys111 has a high probability of undergoing both oxidation and reduction, leading to different results for the functions of this protein, as described below. In addition, Cys111 has become a residue of great interest to better understand the mechanism behind the formation of toxic hSOD1 aggregates observed in ALS cells. Bastow et al. used mutant ySod1 under aging conditions to verify that ySod1 does not aggregate [123]. ySod1 does not have Cys111 (Fig. 3), reinforcing the idea of a possible relationship between the Cys111 residue and the formation of toxic hSOD1 aggregates.

Human SOD1 can be glutathionilated at its four cysteines [107]. Residues Cys57 and Cys146 interact forming the highly stable intramolecular disulfide bond, and closing the β-barrel structure, so that large molecules cannot access these residues. The Cys6 residue in the native form of hSOD1 monomer is buried in the β-barrel structure, so it cannot interact with glutathione; therefore, it does not undergo oxidation by this molecule. As Cys111 is close to the dimeric interface, how does the glutathionylation of this residue affect the stability of the hSOD1 dimer, without affecting the structure and stability of the modified hSOD1 monomer [124]?

Redler et al. demonstrated, using hSOD1 purified from *S. cerevisiae* cells, that Cys111 glutathionylation in a SOD1WT and also in the A4V mutant destabilizes the dimer by decreasing the monomer association rate, and not by the dimeric dissociation kinetics [125]. This raises two hypotheses about how the glutathionylation of Cys111 can lead to decreased formation of the SOD1 dimer: (a) the formation of the bond

between the thiol group of Cys111 and glutathione leads to a steric impediment at the dimeric interface, inhibiting the formation of hydrophobic interactions between hSOD1 monomers; (b) glutathione generate a structural rearrangement of the dimeric interface, to allow entry of this molecule, but as a consequence, inhibits the formation of interactions between the residues at the dimeric interface [125]. This modification indulges an increase in the concentration of stable SOD1 monomers, which can lead to the formation of oligomers and inclusions of hSOD1 observed in ALS cells. However, studies have already shown that glutathionylation alone probably does not lead to the development of the disease, mainly because not all hSOD1 mutants that form inclusions have high levels of glutathionylation [124]. Probably, other changes must occur, in parallel with glutathionylation that contribute to the development of the disease.

Although these results indicate a possible harmful role for Cys111 glutathionylation, some studies indicated the opposite. Using *Saccharomyces cerevisiae* cells deficient in yeast Sod1 and expressing hSOD1WT or hSOD1A4 V, our group demonstrated that glutathionylation can serve as a protector of Cys111 when cells are under oxidative stress [96]. Using yeast cells deficient in glutathione synthesis and expressing hSOD1, we confirmed that after cellular aging the levels of oxidative damage increased and no dysmutasic superoxide activity was detected, suggesting the importance of hSOD1 glutathionylation to exercise its antioxidant role. Similar results were observed when we submitted yeast cells deficient in the enzyme glutaredoxin, which is responsible for reducing the disulfide bond between the thiol group of cysteine and glutathione, and expresses hSOD1, under cell aging conditions [96]. These results suggest that the glutathionylation of hSOD1 plays an important role during recovery from oxidative stress. Glutathione binds to the Cys111 thiol group of hSOD1, stabilizing the monomer and protecting this residue from possible damage that can occur during oxidative stress. Once finalized, glutaredoxin breaks that glutathione-Cys111 bond, and with the steric impediment over, the stable hSOD1 monomers can interact, dimerize, and activate hSOD1, becoming an antioxidant. Similar results were observed with yeast cells expressing hSOD1 under menadione-induced oxidative stress [126].

Cysteinylated in Cys111 can protect hSOD1 structure against oxidative damage and aggregation [111], forming an disulfide bond between the unpaired Cys111 and molecules with free cysteines, protecting the free thiols during oxidative stress. Cys111 is localized in the homodimeric interface, and some authors have define it as an important residue to regulate SOD1 stability and dimerization [27] during oxidative stress and aging [127]. The modification also protects hSOD1 against the hydrogen peroxide formed during the dismutation of the O<sub>2</sub><sup>-</sup> radicals by hSOD1 [128].

#### 4.2.6. Other modifications in lysines: ubiquitination and glycosylation

Ubiquitination is a posttranslational modification that regulates cellular processes related to repair such as degradation of misfolded proteins (by the proteasome or JUNQ), DNA repair, and cell division [129], as well as signaling of cellular pathways related to transcription, translation, transport, and apoptosis [130]. This broadly important modification for the maintenance of homeostasis in eukaryotic cells has been related to some human diseases, including neurodegenerative diseases [129].

Ubiquitination is characterized by the covalent attachment of a ubiquitin (a 76-residue polypeptide highly conserved in eukaryotes) to the ε-amino group of residues that need to be signaled for some type of repair, such as hSOD1 [129]. Studies have demonstrated that hSOD1 undergoes ubiquitination at Lys136 [131] and can be ubiquitinated in other lysines not yet characterized [111]. Coupling of the ubiquitin group to hSOD1 can occur by different ubiquitin ligases, such as Dorfin (cytoplasmic), NEDL1, and MITOL (mitochondrial) [111]. All of these ligases recognize and ubiquitinate hSOD1 mutants, either directly (Dorfin and MITOL) [132,133] or through a complex with another protein (NEDL1 forms a complex with TRAPδ before binding to hSOD1 mut)

[134].

Defects in the degradation process of unfolded proteins signaled by ubiquitination can lead to an exacerbated accumulation, forming the clusters observed in neurodegenerative diseases [130]. Studies with ALS cells found that the accumulation of ubiquitinated proteins in the observed inclusions can decrease the demand for free ubiquitin, preventing other unfolded proteins from being displaced to the proteasome for the degradation process. This rupture in the ubiquitin-proteasome system can lead to the loss of essential cellular functions as well as a decrease in cell survival [130]. In addition, studies working with hSOD1A4 V have elucidated that the ubiquitination of the mutants occurs before the formation of the inclusions, and that cells containing this mutation have decreased proteasome activity, which may be directly related to the accumulation of ubiquitinated hSOD1A4 V inclusions observed in cells fALS [130].

As previously mentioned, some post-translational modifications of hSOD1 can lead to the loss of its enzymatic capacity, such as the glycosylation of some residues of lysine. *In vitro* studies have demonstrated that hSOD1 can be glycosylated at Lys3, Lys9, Lys 30, Lys 36, Lys122, and Lys128 sites [27]. Non-enzymatic glycosylation, also known as glycation, is an irreversible process that occurs spontaneously when intermediate sugars from the glycolytic pathway end up interacting with the amino groups of biomolecules [135]. These sugars are known as advanced glycation end products (AGEs) [109]. Since they interact with biomolecules, AGEs can form a crosslink, affecting their functions [136], solubility, and resistance to proteases [27]. In hSOD1, the glycation of the Lys122 and Lys128 sites leads to a more dramatic inactivation of enzymatic activity when compared to the other glycated lysine [111].

## 5. SOD1: far beyond fALS

Human SOD1 is widely known to be an important protein in the antioxidant system. In 1993, Rosen et al. discovered that hSOD1 presents single point mutations associated with familial cases of amyotrophic lateral sclerosis (fALS) [12]. The principal hallmark of fALS is the presence of hSOD1 aggregates in the spinal cord of patients [137]. However, chemically modified hSOD1, with similar toxic properties as hSOD1 mutants, was found in the spinal cord of sporadic ALS (sALS) patients [138]. Recent studies demonstrated that hSOD1 is overexpressed in some types of cancer, suggesting that this protein could be a target for cancer therapy [11,139]. hSOD1 also seems to play an important role in other neurodegenerative diseases, such as parkinsonism disorders, by interaction with DJ-1 and α-synuclein (aSyn), two key proteins in Parkinson disease (PD) [140,141]. Therefore, we will explore the importance of studying the mechanisms involved in hSOD1 pathology and how this could help in the development of novel therapies for different diseases.

### 5.1. hSOD1 mutants and fALS

ALS is a progressive neurodegenerative disorder that causes loss of motor neurons in spinal cord, brain, and brainstem with an annual incidence between 0.6 and 3.8 per 100,000 people [142]. The familial form affects approximately 10% of the total cases of ALS [143]. Meanwhile, mutations in hSOD1 coding gene represents 20% of all fALS cases [144]. More than 180 mutations in hSOD1 have been found and associated with fALS, including single point mutations, truncations, deletions, and insertions in protein [56]. The hallmark of hSOD1-associated fALS is the presence of hSOD1 protein inclusions in motor neurons, which may indicate a correlation between mutations and the destabilization in the native structure of hSOD1. A variety of studies have demonstrated that hSOD1 mutants enhance the population of monomeric/misfolded species, leading to an increase in the aggregation propensity [145–147].

The toxicity of misfolded and aggregation-prone species of hSOD1

seems to be related to dysfunction of stress granules (SGs) [148,149]. Although the function of SGs has not been well established, they may play an important role in the regulation of protein synthesis in response to different stress conditions, apoptotic signaling, and degradation [150,151]. Mateju et al. found that a ALS-associated mutant of hSOD1 accumulates in the interior of SGs over time, suggesting an aging related mechanism of hSOD1 toxicity [148]. As expected, hSOD1 enzymatic function is also affected by mutations, which indicates a synergy between gain and loss of function [59,152,153].

Interestingly, hSOD1-associated fALS is characterized mostly by autosomal-dominant missense mutations [154]. Different studies in mice have shown that coexpression of WT and mutants accelerates the progress of the disease [155,156]. Recently, our group demonstrated that coexpression of hSOD1 WT and mutants leads to hSOD1 heterodimerization in yeast and cell models (Fig. 4) [58]. Heterodimers were more toxic and formed more inclusions induced by aging than homodimers of mutants (Fig. 4) [58]. Heterodimerization seems to increase the stability of hSOD1 mutants [157]. In addition, the more stable the heterodimer, the shorter the survival time of patients with fALS [158]. These results demonstrate the importance of the native structure of hSOD1 stability under different conditions and the formation of heterodimers when studying hSOD1-associated fALS.

## 5.2. SOD1 chemically modified and sALS

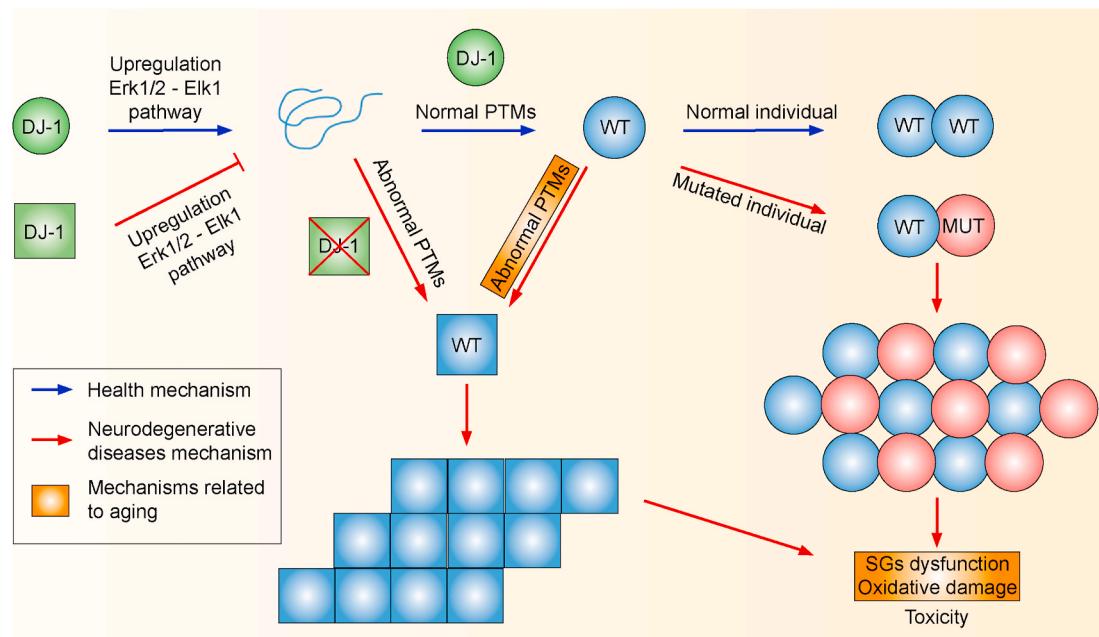
The relationship between SOD1 and fALS is well established due to the genetic factor; however, hSOD1 appears also to play an important role in sALS. As already explained in this work, stability and function of hSOD1 structure depend on different and specific posttranslational modifications (PTMs). In some cases, the pattern of PTMs can be changed or hSOD1 can experience unusual modifications that could lead to destabilization of its native structure and formation of misfolding and insoluble aggregates (Fig. 4) [159–162]. The oxidative damage appears to be related with the formation of toxic species, because hSOD1 WT can

gain similar toxicity to SOD1-associated fALS mutants when oxidized [138,163]. A hyper-oxidized and toxic form of hSOD1 WT was found in a subset of patients with bulbar onset [164]. To explain how the oxidation mechanism leads to formation of toxic species, Petrov et al. demonstrated that single residue oxidation decreases the dimer and monomer stability of hSOD1, which could lead to an increase in misfolding species and aggregation propensity, similar to the effect of hSOD1-associated fALS mutants [118]. In *S. cerevisiae*, the oxidation of residues important for ySod1 stability was cause by aging and promoted the formation of soluble aggregates [122].

Oxidation is not the only PTM that seems to be related with sALS. Despite the inhibition of amyloid aggregate formation, hSOD1 WT glycation increases the protein toxicity in cell models [109]. Polykretis et al. demonstrated that methylglyoxal, a highly reactive by-product of glucose metabolism, can promote *in vitro* glycation of apo hSOD1 and formation of misfolding and oligomeric SDS-stable species of hSOD1 WT [136]. Furthermore, endoplasmic reticulum (ER) stress, an important machinery in proteostasis, seems to be correlated with formation of misfolded and oligomeric forms of hSOD1 in transgenic mice, which indicate that ER stress could be a potential risk factor for sALS [165]. Finally, our work sheds light in the importance of studying the role of hSOD1 also in sALS and what could regulate and initiate these unnatural modifications in hSOD1 structure.

## 5.3. hSOD1 and cancer

Cancer cells are known to have high levels of reactive oxygen species (ROS), which increase their need for the antioxidant system [166,167]. Interestingly, hSOD1 is overexpressed in a variety of cancers, and its inhibition leads to impaired mitochondrial function in breast cancers [22,139]. Human SOD1 overexpression can activate the mitochondrial unfolded protein response (UPRmt), a key player in cytoprotective response, and maintain the ROS levels appropriate to a cancer cell [11,168]. In addition, hSOD1 knockdown or inhibition, suppresses the



**Fig. 4. Proposed mechanism of SOD1 aggregation in neurodegenerative disorders.** In healthy individuals (blue arrows), DJ-1 regulates SOD1 expression by Erk1/2-Elk1 pathway and, together with normal PTMs, also acts as a copper chaperone to promote holoSOD1 monomer formation, thereby forming SOD1 dimer. In neurodegenerative disorders (red arrows), mutations in DJ-1 associated with PD may impair SOD1 expression and increase apoSOD1 population through loss of copper chaperone function, leading to oxidative stress and aggregation. SOD1 is subjected to abnormal PTMs before and after monomer maturation, which could enhance aggregation-prone species population. These aggregates are related to sALS cases. Heterozygous mutations in SOD1 gene associated with fALS lead to heterodimer formation, which increases SOD1 aggregation. Both SOD1 sALS and fALS aggregates cause SGs dysfunction, oxidative damage, and cellular toxicity. Orange boxes indicates process related to aging.

growth of non-small-cell lung cancer (NSCLC) and leukemia [22,169], supporting the role of hSOD1 as a pro-oncogenic protein. Gomez et al. demonstrated that hSOD1 overexpression is essential for tumor formation and hypothesized that the mechanism of overexpression is by the mTORC1 pathway [170]. In cancer, cells suffer starvation in early formation stages and under these conditions, mTORC1 is inactivated, leading to hSOD1 overexpression [21]. Notably, hSOD1 inhibition induces apoptosis in cancer cells, which indicates that hSOD1 is a potential novel target for cancer therapy [170,171].

#### 5.4. *hSOD1 and other neurodegenerative disorders*

Human SOD1 is known to be involved in ALS pathogenesis, and this protein seems to be related to the development of other neurodegenerative diseases, such as Parkinsonism disorders. DJ-1, a key protein in PD pathology, whose mutations cause loss of function and lead to the death of dopaminergic neurons [172]. It upregulates hSOD1 expression through the Erk1/2-Elk1 pathway [173] and can also act on hSOD1 activation by a copper chaperone for SOD1 (CCS) independent pathway [140]. Thus, indicating that there is a synergy between DJ-1 and hSOD1 in neuroprotection against oxidative stress (Fig. 4).

Recently, single nucleotide polymorphism was discovered in the hSOD1 coding gene (rs2070424 A/G) associated with an increase in genetic susceptibility of PD [174]. Furthermore, hSOD1 appears to interact with  $\alpha$ -synuclein (aSyn), the principal constituent of Lewy Bodies in PD. Helferich et al. demonstrated that aSyn binds to hSOD1 in different models and accelerates its oligomerization without affecting hSOD1 enzymatic activity [141]. In addition, misfolded hSOD1 accumulation was found in regions affected with neuronal loss in PD brain [10]. Although these aggregates are not associated to hSOD1-associated fALS mutations, they exhibit evidence of misfolding and metal deficiency as well as similar neurotoxic effects [175]. These results suggest a similar mechanism among hSOD1 aggregates in ALS and PD, which could lead to the development of novel therapeutic targets for both diseases.

### 6. Models to study the importance of hSOD1 to health and perspectives

Advances in molecular biology and genetic engineering have facilitated the modulation of cellular aspects to study and identify how hSOD1 could act under different conditions. These models include *in vitro* biochemical systems, unicellular organisms, small-animals, and rodent models. In this section, we will discuss the advantages of each of these models mentioned above and how they are being used for important discoveries about the relevance of hSOD1 to health.

The *in vitro* biochemical system model is commonly used as the first step to understand important behaviors of hSOD1 that are relevant to diseases mentioned in topic 5. As a low-cost model, *in vitro* assays provide a controlled environment where hSOD1 properties can be studied without interference of other proteins or small molecules. Although the results do not always reflect the reality in the human body, they serve as a base for future studies in more complex models. For instance, using a biochemical system *in vitro* is the simplest way to study the propensity of hSOD1 WT misfold/aggregate and compare this with hSOD1-associated fALS mutants, to identify an interaction between two proteins, or to analyze the capacity of a small molecule to promote or inhibit hSOD1 aggregation. For instance, Polykretis et al. applied *in vitro* assays to determine that a glycation agent could promote hSOD1 oligomerization, suggesting a mechanism for hSOD1 aggregation in sALS [136]. Human cells, such as H4 and HEK cells, are also biochemical systems extensively used to study hSOD1 aggregation and toxicity, especially with the advances in CRISPR technology that can knockdown cells in the SOD1 gene [58].

The *S. cerevisiae* model is widely used to modulate a range of diseases, and studies using this model have already received a good number

of Nobel Prizes in Chemistry and Medicine. Important roles of hSOD1 discussed in topic 5, such as the mechanism of hSOD1 overexpression in cancer and the higher toxicity of hSOD1 heterodimers in fALS, were studied in *S. cerevisiae* [21,58]. Although this model does not fully replicate the complexity of the human body and all the aspects involved in diseases like cancer or ALS, it remains an important tool for identifying the molecular mechanisms associated with these disorders.

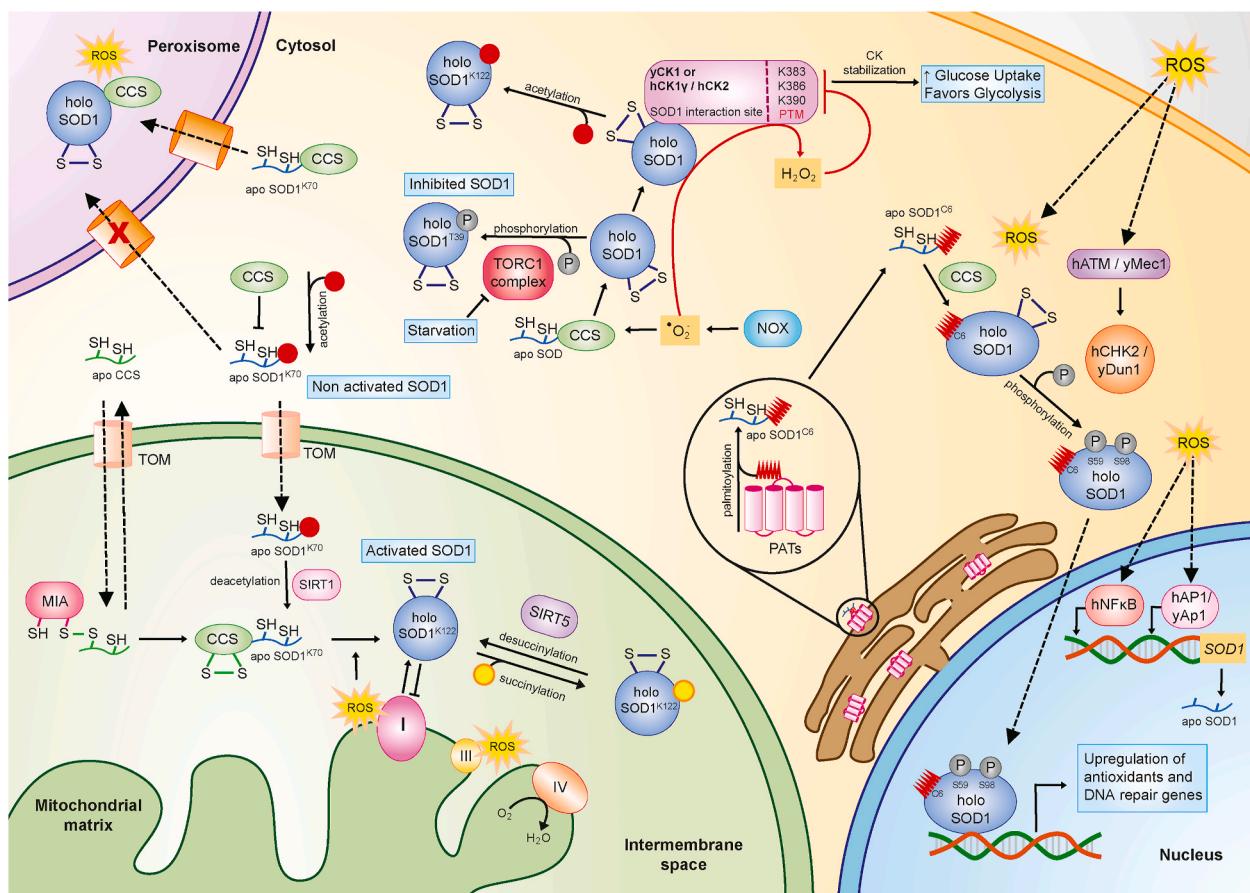
The lack of motor neurons (MN) in yeast and cellular models are a crucial limitation. The presence of these neurons and the lower cost compared to rodent models makes small animals, such as the worm *Caenorhabditis elegans* and the fly fruit *Drosophila melanogaster*, relevant models for neurodegenerative disorders associated with hSOD1. *C. elegans* has 35% homologous genes with humans, while *D. melanogaster* presents 70%. Like *S. cerevisiae*, the genome of both have been screened, and a library of mutants associated with hSOD1 pathology are available for researchers throughout the world. Small-animal models can simulate motor phenotype, protein aggregation, mitochondrial dysfunction, etc. [176,177], which makes them an important base for generating hypotheses about the role of hSOD1 in different neurodegenerative disorders.

Rodent models are the most used model to study human diseases in general and for hSOD1-associated disorders. Although mice cannot modulate aspects of ALS that are not related to hSOD1 mutations or overexpression, studies have shown the increase in toxicity of SOD1 mutants when coexpressed with hSOD1 WT, which is an important breakthrough for fALS [155,156]. In addition, mice models for cancer study reveal that hSOD1 overexpression is a key factor in tumor formation [170], and models for PD demonstrated the interaction between hSOD1 and aSyn [141]. Despite not having its entire genome screened and the higher maintenance cost compared to other models, the 100% of homologous genes and the advances in genetic engineering turns this model into an essential step to understand the roles of hSOD1 and discover novel targets for therapy against hSOD1-associated diseases.

Recently, the induced pluripotent stem cells (iPSCs) arrived as the greatest hope to modulate both fALS and sALS, because now it is possible to generate cells derived from patients with ALS, independent of the presence of mutations [178]. This model can be differentiated into the specific cells of interest, including spinal motor neurons, extremely relevant to ALS [179]. On the other hand, the generation of upper motor neurons has not been elucidated yet [180]. The progress in CRISPR-9/Cas technique enables the correction of gene mutations in iPSCs derived from patients and the appropriate comparison between model and individual [181]. Despite the necessity for improvements in the iPSC model, especially in mimicking aging aspects of neurodegenerative disorders, their ability to modulate not only diseases with gene mutations, but also mechanisms involved in sporadic cases, increases the expectations around this model.

### 7. Concluding remarks

The function and native conformation of hSOD1 depend on PTMs, including the formation of intramolecular disulfide bond between Cys57 and Cys146 and metallation [110,27]. These processes depend on the chaperone CCS. SOD1 activation also depends on aerobic conditions. According to Fetherolf et al. ROS modulate SOD1 activity [182]. Human SOD1 is found in a variety of cellular compartments, such as cytosol, mitochondrial intermembrane space (IMS), and peroxisome, acting as an antioxidant enzyme and in signaling, as well as in the nucleus, regulating the expression of genes involved with oxidative stress response [14–16]. An ever increasing number of PTMs are being found in hSOD1 structure and some of them are related to hSOD1 trafficking and different roles, as mentioned in topic 4.2 [108,27]. The mechanisms of PTMs, despite being treated independent of each other, are interconnected and constantly occur in cells in response to different signals, especially, in the case of hSOD1, to oxidative stress [183–185]. Herein, we illustrate an integrated mechanism of SOD1 regulation (Fig. 5).



**Fig. 5. Integrated mechanism of SOD1 regulation.** SOD1 is a critical enzyme in maintaining ROS homeostasis in different cell compartments. SOD1 gene is highly and ubiquitously expressed, although its expression can be further induced by ROS-responsive transcription factors, such as NF- $\kappa$ B and AP1 [54]. The activation of apoSOD1 is also dependent on ROS and mediated by the chaperone CCS [182]. SOD1 is highly abundant in the cytoplasm, where NADPH oxidases (NOX) are the major contributors to superoxide levels. The product of NOX might activate SOD1 and be converted into peroxide by SOD1. The interaction of SOD1 with a casein kinase, which regulates glucose and nutrient sensing, promotes the stabilization of the kinase. SOD1 peroxide product reacts with the  $\epsilon$ -amino group of K383, K386, and K390 of the casein kinase, inhibiting their ubiquitination (PTM), which in turn stabilizes the kinase and favors aerobic glycolysis, an important mechanism in cancer proliferation [19]. One hypothesis for the disruption of the SOD1-casein kinase interaction is the K122 acetylation of hSOD1 [27], promoting casein turnover. In cytosol, mTORC1 inhibits SOD1 activity through phosphorylation at T39 in response to nutrients, promoting cell proliferation (in yeast, Sod1 is phosphorylated at S38) [21]. Starvation inhibits mTORC1, which activates SOD1 activity, preventing oxidative damage and increasing survival. SOD1 is also necessary in peroxisome. The non-acetylated K70 apoSOD1 interacts with CCS, leading to SOD1 transport to peroxisome, where it is activated to eliminate ROS [14]. Complexes I and III are the major source of ROS, and part of them are released into intermembrane space (IMS). SOD1 acetylation at K70 leads to its dissociation from CCS, which might enable SOD1 transport to IMS through TOM. Acetylation of the K70 residue disrupts hSOD1 interaction with CCS and extinguishes hSOD1 enzymatic activity [108], suggesting a role of this PTM in modulation of hSOD1 transport to mitochondrial IMS or peroxisome. At IMS, SOD1 is deacetylated by SIRT1 and activated by CCS, which is SOD1-independently transported by TOM and activated by MIA [52,186]. Once in the activated form, SOD1 is trapped in the mitochondrial IMS, where, besides eliminating ROS, SOD1 interacts with complex I to regulate the respiratory rate and ROS production. SOD1-complex I interaction is modulated by SIRT5, which desuccinylates SOD1 at K122 and maintains the protein active [113]. SOD1 is transported to the nucleus in response to oxidative stress, where it acts as a transcription factor, inducing antioxidants and DNA repair genes expression [16]. Palmitoylation at C6 by Protein Acyl Transferases (PATs) is required for nuclear localization of SOD1 and avoids denaturation of apoSOD1 [110,116]. Although the mechanism by which palmitoylation regulates hSOD1 trafficking to the nucleus is not fully elucidated, it seems to play an important role in the formation of hSOD1 intramolecular disulfide bond by directing cytoplasmic apo hSOD1 to membrane [110]. The transport of SOD1 to the nucleus also depends on S59 and S98 phosphorylation, a PTM conserved in yeast and humans [16]. Human SOD1 phosphorylation involves ATM, while yeast Sod1 phosphorylation seems to involve Mec1 and Dun1.

In addition, hSOD1 mutations associated with fALS cases, despite being related to loss of enzymatic function and gain of toxic properties through misfolding and aggregation, appear also to be relevant in hSOD1 mislocalization. Our group demonstrated that both homodimeric and heterodimeric hSOD1 mutants reduce the nuclear localization in response to oxidative stress, when compared to hSOD1 WT [58,59]. However, hSOD1 mutations seem not to affect IMS localization, since the transport to IMS happens before maturation [186]. Moreover, the same work proposed that these mutations are trapped inside the mitochondria due to misfolding and aggregation, leading to bioenergetic defects [52,186]. With this review, we expect to assist not only for a better understanding about how PTMs modulate hSOD1 function and

localization, but also provide valuable information about which events could trigger hSOD1 pathology, as well as the potential role of these PTMs in therapy and early diagnosis of hSOD1 associated disorders.

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