

## Research Article

# Peroxiredoxin 2 (*PRDX2*), an antioxidant enzyme, is under-expressed in Down syndrome fetal brains

M. F. Sánchez-Font<sup>a</sup>, J. Sebastià<sup>b</sup>, C. Sanfeliu<sup>b</sup>, R. Cristòfol<sup>b</sup>, G. Marfany<sup>a</sup> and R. González-Duarte<sup>a,\*</sup>

<sup>a</sup> Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, 08028 Barcelona (Spain), Fax: + 34 93 4110969, e-mail: rgonzalez@ub.edu

<sup>b</sup> Departament de Farmacologia i Toxicologia, Institut d'Investigacions Biomèdiques de Barcelona, CSIC, IDIBAPS, Rosselló 161, 08036 Barcelona (Spain)

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**Abstract.** Suppression subtractive hybridization performed on Down syndrome (DS) versus control fetal brains revealed differential expression of peroxiredoxin 2 (*PRDX2*), mapped at 13q12. Peroxiredoxins are antioxidant enzymes involved in protein and lipid protection against oxidative injury and in cellular signalling pathways regulating apoptosis. The under-expression of *PRDX2* observed in DS samples was confirmed by real-time PCR (0.73-fold). To test whether decreased expression is associated with enhanced sensitivity of DS neurons to reactive oxygen species, we down-regulated

*PRDX2* through stable transfections of SH-SY5Y neuroblastoma cells with antisense constructs of the complete *PRDX2* coding sequence. In addition, we over-expressed *SOD1* and compared the effects of the two genes on cell viability. Cells transfected with either construct showed similar sensitivity to oxidative stress in addition to increased apoptosis under basal conditions and after treatment with oxidative cytotoxic agents. This suggests that the decreased expression of *PRDX2* may contribute to the altered redox state in DS at levels comparable to that of the increased expression of *SOD1*.

**Key words.** Down syndrome; peroxiredoxin; SOD1; oxidative stress; suppression subtractive hybridization; differential expression.

## Introduction

Down syndrome (DS), the most common chromosomal aneuploidy in liveborn infants [1, 2], is caused by total or partial trisomy of chromosome 21. The complete sequence of this chromosome has been recently reported [3], but the relationship between specific DS phenotypic features and the gene dosage imbalance associated with the trisomy remains a challenging issue. This relationship could be either direct or indirect, because the extra gene copies in DS may lead to a cascade effect, involving additional genes not necessarily located on chromosome 21.

Concerning the phenotype, a broad spectrum of abnormalities related to oxidative stress, including mental retardation, early onset Alzheimer's disease and premature ageing, has been associated with DS. In fact, several genes on chromosome 21 are involved in the cellular redox state, namely the Cu/Zn superoxide dismutase (*SOD1*), cystathione- $\beta$ -synthase (*CBS*), carbonyl reductase (*CBR*), Down syndrome critical region gene 1 (*DSCR1*) and the heat-shock protein family member *STCH* [reviewed in ref. 4]. Deregulation of these genes may alter the levels of intracellular reactive oxygen species (ROS), produced during ordinary metabolic activities of aerobic organisms, and generate oxidative stress. Significantly, some ROS are involved in signalling

\* Corresponding author.

pathways but, when cell defences are overcome, ROS turn into powerful damaging agents that lead to apoptotic or necrotic cell death [5]. A battery of antioxidant enzymes prevents the harmful effects of ROS on DNA, proteins and lipids [6].

To find candidates contributing to the DS phenotype, we searched for differentially expressed genes by performing suppression subtractive hybridization (SSH) on DS versus control fetal brain samples. Following this approach, we showed that peroxiredoxin 2 (*PRDX2*) was under-expressed in DS fetal brains with respect to controls. *PRDX2* is a human homologue of yeast thioredoxin peroxidase (*TPX*) [7–14], also named thioredoxin-dependent peroxide reductase 1 (*TDPX1*) or natural killer-enhancing factor-B (*NEKF-B*). It is located on chromosome 13q12 [15] and is expressed in human neurons but not in glial cells [16].

Peroxiredoxins (PRDXs), initially characterized in yeast, constitute a family of antioxidant enzymes with no homology with conventional antioxidant proteins [7, 9, 12]. More than a hundred homologues have been described from bacteria to mammals, sharing highly conserved protein domains [17, 18]. They were first named thioredoxin peroxidases because they reduce  $H_2O_2$  to water using thioredoxin as an intermediate electron donor, but were later renamed, because some PRDXs do not require thioredoxin [9]. PRDXs play a key role in several cellular functions including protein and lipid protection against oxidative injury [12–14], cell proliferation, differentiation [19] and intracellular signalling pathways regulating apoptosis [20, 21]. Increased cellular levels of antioxidant enzymes confer protection against apoptosis. In this respect, cultured cells over-expressing *PRDX2* are more resistant to apoptosis caused by serum deprivation, hydrogen peroxide, ceramide and etoposide [22]. *PRDX1* and *PRDX2* over-expression also protect thyroid cells from  $H_2O_2$ -induced apoptosis [21].

To test whether the decreased expression of *PRDX2* in DS brains is related to the reported oxidative stress in DS cultured neurons, we achieved *PRDX2* under-expression through stable transfections of SH-SY5Y neuroblastoma cells with antisense constructs of the *PRDX2* coding sequence. We also over-expressed *SOD1*, which is located at 21q22.1 and whose activity is increased 1.3- to 1.6-fold in DS. We then compared the effects of the altered expression of either gene on basal cell viability and resistance against oxidative injuries produced by several cytotoxic agents. Cells transfected with either construct showed increased apoptotic cell death under both basal conditions and oxidative stress. Overall, the *PRDX2* antisense-expressing clones showed similar or slightly higher sensitivity to oxidative injuries than those over-expressing *SOD1*.

## Material and methods

### Samples

Half brains (including half cerebellum) from 19 male fetuses obtained from therapeutic abortions were collected for this study. Ten of these samples were diagnosed as trisomic for chromosome 21 by amniocentesis (mean gestational age of  $20.11 \pm 1.18$  weeks), while the remaining 9 did not show any chromosomal aberration and were used as controls (mean gestational age of  $20.11 \pm 1.56$  weeks). Fresh tissue was directly frozen in liquid nitrogen. Initially, 6 samples (3 disomic vs 3 trisomic) were used for SSH. Differential expression was confirmed by real-time PCR quantification using 13 additional samples (6 disomic vs 7 trisomic). The use of all human material in this research was approved by the Ethics Committee of the University of Barcelona.

### mRNA extraction, cDNA synthesis and SSH

Total RNA was extracted from six samples (three DS vs three control) as described elsewhere [23]. PolyA<sup>+</sup> RNA was obtained using biotinylated oligo(dT) (Promega) and streptavidin-magnetic beads (Dynal), and retrotranscribed using MMLV-RT (Promega), following the manufacturer's instructions. The cDNA second strand was obtained using the enzyme cocktail and protocol provided by the PCR-SELECT cDNA subtraction kit (Clontech). SSH was performed on DS and control mRNAs using the PCR-SELECT cDNA subtraction kit (Clontech) according to the manufacturer's instructions with minor modifications. Differentially expressed cDNAs were cloned and sequenced after two rounds of subtractive hybridization to enrich the sample and two rounds of nested PCR amplification (20 primary and 30 secondary PCR cycles) using the Advantage KlenTaq polymerase (Clontech).

### Quantification of differentially expressed genes by real-time quantitative PCR

Total RNA was obtained using the ABI PRISM 6700 Automated Nucleic Acid Workstation (Applied Biosystems) and subsequent RT-PCRs were produced using the Taqman Reverse transcription reagents (Roche Molecular Systems). Quantitative PCRs were performed with the Universal Master Mix (Applied Biosystems) and analysed by the ABI PRISM 7700 sequence detection system (Applied Biosystems). The primers and Taqman probe (using FAM as reporter and TAMRA as quencher) were designed using the Primer Express software. Relative expression values of *PRDX2* with respect to *GAPDH* and  $\beta$ -*tubulin* controls (primers and probe from Applied Biosystems) were obtained following the manufacturer's instructions.

### DNA constructs

The pBI-*PRDX2* antisense-enhanced green protein (EGFP) and the pBI-*SOD1* sense-EGFP constructs were obtained by cloning the corresponding cDNA coding sequences (from ATG to the stop codons) into the pBI-EGFP vector (Clontech) in the antisense and sense direction, respectively.

### Cell culture and transfection

Human neuroblastoma SH-SY5Y cells (European Collection of Cell Culture) were grown under the following standard conditions: MEM-F12 (Gibco, Invitrogen) supplemented with 2  $\mu$ M glutamine, 1% non-essential amino acids, 50  $\mu$ g/ml gentamicin and 10% Tet-system approved fetal calf serum (Clontech). DNA constructs were electroporated into cells (40  $\mu$ g for  $5 \times 10^6$  cells) using a Gene Pulser II (950  $\mu$ F, 300 V; Bio-Rad). Tet-on cell lines were obtained by transfection with the pTet-on vector (Clontech) and subsequent selection with G418 (0.5 mg/ml) (Gibco, Invitrogen) after 2–3 days. A second round of transfection was performed on selected pTet-on cell lines with a 1:40 mix of pTK-hygromycin empty vector and either pBI-*PRDX2* antisense-EGFP or pBI-*SOD1* sense-EGFP. After 2–3 days in culture, co-transfected cells were selected by adding G418 (0.5 mg/ml) and hygromycin (0.150 mg/ml) (Gibco, Invitrogen). Resistant and fluorescent clones were isolated, expanded and used in further experiments. The initially selected pTet-on clones were used as controls. The expression of the sense and antisense constructs was induced by adding tetracycline (2  $\mu$ g/ml) onto the media.

### SOD1 activity measurements

Total SOD activity was determined in cell homogenates by the xanthine/xanthine oxidase method using a commercial kit (Ransod, Randox).

### Western immunodetection

Cells ( $5 \times 10^5$ ) from each selected clone were collected, pelleted by centrifugation, rinsed once with PBS and resuspended in 75  $\mu$ l of total protein extraction buffer (150 mM NaCl, 10 mM Tris HCl pH 7.4, 1 mM EDTA, 1% NP40 plus a cocktail of protease inhibitors). After 10 min on ice, samples were centrifuged for 15 min at 4 °C to separate soluble protein extracts from cellular debris. Protein extracts were quantified following the Bradford method (and further confirmed by Coomassie staining of a replica gel), loaded (5  $\mu$ g/lane) onto a 10% SDS-polyacrylamide gel, electrophoresed, semi-dry transferred onto a PVDF membrane, immunodetected with both a polyclonal antibody (1:5000) raised against PRDX2 (kindly provided by Dr H. Z. Chae) and a  $\beta$ -tubulin commercial monoclonal antibody (1:5000), and detected with ECL (Amersham Pharmacia). Relative quantification of PRDX2/ $\beta$ -tubulin expression in clones was

performed with the NIH Image 1.62f software. For caspase-3 activity detection, the same general method and conditions were used but for loading, 30  $\mu$ g of protein extract per lane onto a 7.5% SDS-polyacrylamide gel, immunodetection with an anti-fodrin antibody (Chemicon) (1:2000) and chemiluminiscent detection with SuperSignal (Pierce).

### Viability assays

For all experiments, growing cells were harvested after mild trypsinization and seeded at a concentration of  $2$  or  $3 \times 10^5$  cells/ml in 30-mm Petri dishes, 96-well plates or 8-chamber slides (Nunc) in growth medium. Tetracycline (2  $\mu$ g/ml) was added the next day and experiments were performed 24 or 48 h (approximately at 80% of cell confluence) after induction. Basal levels of cell death in the control, *PRDX2* antisense and *SOD1* sense transfected cell lines grown on 96-well dishes were determined by propidium iodide staining (7  $\mu$ g/ml), a fluorescent dye that binds to nucleic acids in dead cells [24]. After 1 h incubation, fluorescence was quantified using a Cytofluor 2350 fluorescence scanner (Millipore) [485 nm (20 nm band-pass) excitation and 530 nm (40 nm band-pass) emission]. Basal levels of cell death were calculated as a percentage of the maximum fluorescence obtained in wells with cells treated with 0.02% Triton X-100. The differential effects of several neurotoxic agents, namely hydrogen peroxide, thimerosal and etoposide (Sigma), on the growth and survival of gene-transfected cells and controls were analysed by the 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay with minor modifications [25]. MTT was added to the 96-well cultures (final concentration, 0.5 mg/ml), 2 h before the end of a 24-h exposure to the toxic agent. After incubation, absorbance was measured at 560 nm with a 620-nm reference wavelength in an iEMS (Labsystems) plate reader. Data (four to seven independent experiments) of basal cell death and response to cytotoxic agents were pooled and expressed as a percentage of controls. The results are given as the mean  $\pm$  SE. Statistical analysis was performed with ANOVA procedures followed by Duncan's multiple-range test at the significance level  $p < 0.05$  (SPSS software).

Determination of the enzymatic activity of lactate dehydrogenase (LDH) released into the culture medium by damaged cells was performed as previously described [26] and used as a reference of cytotoxicity. Propidium iodide-stained and TUNEL processed cells (see below) cultured in chamber slides were also examined under the microscope.

### Caspase-3 immunostaining and activity quantification

Transfected cells and controls were immunostained for the presence of cleaved caspase-3 after growth under standard conditions or under treatment with hydrogen peroxide or

thimerosal. Cells on coverslips were fixed, and immunodetected with a rabbit polyclonal antibody specific for cleaved caspase-3 (Cell Signalling) (1:100) and a biotinylated goat anti-rabbit IgG (Vector) (1:200), followed by detection with the Vectastain ABC-kit (Vector).

Protein extracts of each selected clone were obtained from untreated and treated (after 3 h exposure to 100  $\mu$ M hydrogen peroxide) cells ( $1 \times 10^6$ ) as in Western immunodetection but for the lysis buffer (200 nM HEPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% sucrose, 1 mM PMSF and 1% NP-40 at pH 7.4). Caspase-3 activity was measured in a 96-well plate. Twenty-five micrograms of protein extract were loaded per well. Three values were obtained for each extract: (i) caspase-3 activity on the DEVD-AFC substrate (final concentration 10  $\mu$ M); (ii) the same as (i) but adding DEVD-FMK (an inhibitor of caspase-3 activity) diluted in DMSO (final concentration 10  $\mu$ M), and (iii) a control value of (ii) by adding DMSO instead of inhibitor to the lysis buffer. After incubating the 96-well plate containing all protein extracts and reagents (except the substrate) for 15 min at 37  $^{\circ}$ C, the substrate DEVD-AFC was added to each well. For each sample, caspase-3 activity was calculated as  $i - (ii - iii)$ . Fluorescence (excitation  $\lambda$  at 400 nm, emission  $\lambda$  at 505 nm) was quantified at several incubation times (0, 0.5, 1, 3 and 6 h).

#### TUNEL assays

Tunel experiments were performed using the DeadEnd Colorimetric Apoptosis Detection System (Promega). A previous time course quantification of TUNEL-positive cells after hydrogen peroxide and thimerosal treatment at 3, 8, 15 and 24 h was performed and the best results were achieved at 15-h treatment. The percentage of TUNEL-positive cells was analysed in controls and in two transfected clones for each construct, under standard and the specified oxidative stress conditions. Positive cells were counted in five different microscope areas of 100 cells for each sample (two to four samples per clone and per experimental condition). Statistical analysis was performed with ANOVA procedures followed by Duncan's multiple-range test at the significance level  $p < 0.05$  (SPSS software).

## Results

#### Down-regulation of PRDX2 in DS fetal brains

To identify putatively down-regulated genes in DS, SSH was performed on brain tissue samples (half brains) from three DS and three control male fetuses, using an excess of DS cDNAs (driver population) with respect to control cDNAs. The DNA fragments obtained (ranging from 570 to 190 bp) were cloned and sequenced. Most of them corresponded to ribosomal protein genes and unidentified

transcripts which were not further investigated. Differential expression of the remaining genes was assessed by densitometric analysis of Northern blot hybridizations (data not shown). Consistent data on decreased expression associated with DS was only obtained for PRDX2. Further confirmation and quantification was obtained by real-time PCR ( $0.73 \pm 0.08$  fold) on 13 additional samples, 6 disomic and 7 trisomic, using GAPDH and  $\beta$ -tubulin to normalize the values. Statistical significance was determined by the Mann-Whitney test ( $p < 0.05$ ) (fig. 1).

#### Transfection of SH-SY5Y cells and selection of clones

To analyse the effect of PRDX2 under-expression in cultured neuronal cells, an antisense strategy to sequester the endogenous transcript was designed. A SOD1 sense expression construct was also produced to raise the cellular levels of SOD1 enzymatic activity. To prevent possible deleterious effects caused by constitutive expression of the PRDX2 and SOD1 constructs in stably transfected cells, we used the pTet-on inducible system (BD-Clontech).

After a round of transfection, several independent clones were selected and shown to express high levels of rtTA

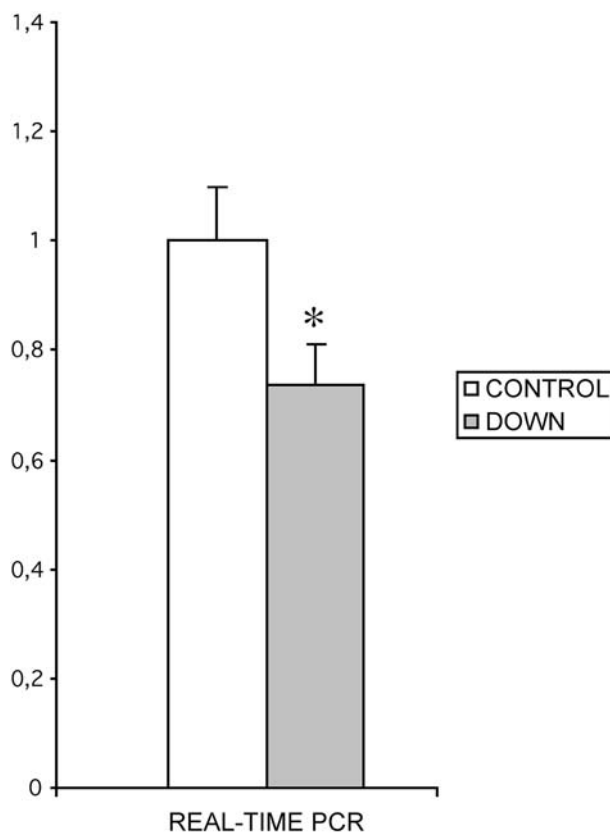


Figure 1. Average differential PRDX2 expression of DS samples with respect to controls (7 trisomic vs 6 disomic) by real-time quantitative PCR. Values in arbitrary units are normalized with GAPDH as an endogenous control. The statistical error is also shown. The asterisk indicates statistical significance according to the Mann-Whitney test ( $p < 0.05$ ).

(assessed by RT-PCR, data not shown). Two of these positive Tet-on cell lines were then transfected with a second plasmid containing a tetracycline-dependent bidirectional promoter (pBI-EGFP; BD-Clontech) that controlled the transcription of both the EGFP reporter and the gene of interest (either the *PRDX2* antisense or the *SOD1* sense constructs). For each construct, between 40 and 50 clones were selected by growth on G418- and hygromycin-supplemented medium. Tetracycline was added afterwards to induce the EGFP reporter. Twenty highly fluorescent cell lines of each type were isolated and maintained. However, low expression levels of EGFP were also detectable in the absence of the inducer, as in recent reports on the regulation efficiency of the Tet-on system [27]. Unless otherwise stated, all experiments were performed with induced cells.

Clones showing low levels of endogenous *PRDX2* (as detected by immunodetection) (fig. 2) or high *SOD1* expression (as detected by enzymatic activity) (table 1) were selected for subsequent analyses.

### Viability studies

Cell clones transfected with either construct showed higher spontaneous mortality than controls under stan-

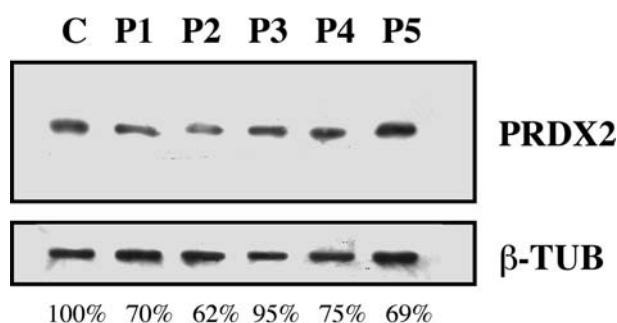


Figure 2. *PRDX2* and  $\beta$ -tubulin immunodetection by Western blot to assess *PRDX2* under-expression in selected clones. C, control cells transfected with pTet-on vector only; P1 – P5, independent clones transfected with *PRDX2* antisense construct (relative under-expression values are indicated as percentages with respect to control).

Table 1. *SOD1* activity in control cell line SH-SY5Y and in *SOD1*-transfected clones.

Cell line	<i>SOD</i> activity (U/mg protein)
Control	4.40 ± 0.54 (5)
S1	17.56 ± 5.27 <sup>a</sup> (4)
S2	12.46 ± 3.25 <sup>a</sup> (3)
S3	19.25 ± 2.28 <sup>a</sup> (4)

Data are mean ± SE values (with the number of independent determinations in parentheses).

<sup>a</sup>  $p < 0.05$ , statistically different from control cell line by Student's *t* test.

dard conditions (see Material and methods), as revealed by propidium iodide staining (fig. 3a–e). Statistical significance of the increased mortality of clones over-expressing *SOD1* ( $F_{3,76} = 5.734$ ,  $p = 0.001$ ) (fig. 3a) or under-expressing *PRDX2* ( $F_{5,155} = 2.611$ ,  $p = 0.026$ ) (fig. 3b) was shown by one-way ANOVA.

The cytotoxicity of hydrogen peroxide, thimerosal and etoposide was tested at two concentrations, previously shown to cause low and moderate cell death in control cultures. The MTT test was used on transfected cells and controls to evaluate the effects on cell proliferation and viability, which was dependant on the construct and the treatment. Overall, transfected clones (with either *PRDX2* antisense or *SOD1* sense) were more sensitive than controls at the highest concentration of the cytotoxic agents. In the *PRDX2* antisense transfectants, two-way (agent concentration and clone) ANOVA showed a significant effect of the toxic agent concentration for hydrogen peroxide ( $F_{2,807} = 1180.16$ ,  $p < 0.001$ ), thimerosal ( $F_{2,432} = 512.63$ ,  $p < 0.001$ ) and etoposide ( $F_{2,438} = 373.84$ ,  $p < 0.001$ ). However, differences between *PRDX2* clones and controls were significant only for hydrogen peroxide ( $F_{5,807} = 22.77$ ,  $p < 0.001$ ) and thimerosal ( $F_{5,432} = 12.998$ ,  $p < 0.001$ ). Moreover, *SOD1* transfectants were also significantly affected by the concentration of hydrogen peroxide ( $F_{2,509} = 1086.83$ ,  $p < 0.001$ ), thimerosal ( $F_{2,295} = 299.23$ ,  $p < 0.001$ ) and etoposide ( $F_{2,424} = 239.49$ ,  $p < 0.001$ ). In this case, differences between *SOD1* clones and controls were significant only for hydrogen peroxide ( $F_{3,509} = 44.78$ ,  $p < 0.001$ ), although there was a significant interaction effect between both factors, clone and concentration, for thimerosal ( $F_{6,295} = 6.34$ ,  $p < 0.001$ ). MTT assay responses to hydrogen peroxide and thimerosal exposure are shown in figures 4a, b and 5a, b, respectively. For each concentration of the toxic agents, sensitivity differed significantly with respect to controls, as shown by one-way ANOVA followed by Duncan's test (figs. 4a, b and 5a, b). Overall, cells transfected with *PRDX2* antisense showed higher sensitivity than those over-expressing *SOD1* to hydrogen peroxide (at all assayed concentrations,  $F_{1,981} = 37.09$ ,  $p < 0.001$ ) and thimerosal (only at the lowest concentration,  $F_{1,177} = 5.70$ ,  $p = 0.017$ ).

The LDH assay and propidium iodide staining also confirmed the MTT results with similar statistical significance (data not shown). After treatment with hydrogen peroxide or thimerosal, cell death increased in *SOD1* sense and *PRDX2* antisense transfected cultures with respect to controls (see results on propidium iodide staining, figs. 4c–e and 5c–e).

After treatment with etoposide, the viability of the transfected cells did not differ significantly from controls (data not shown) and, thus, they were not further studied.

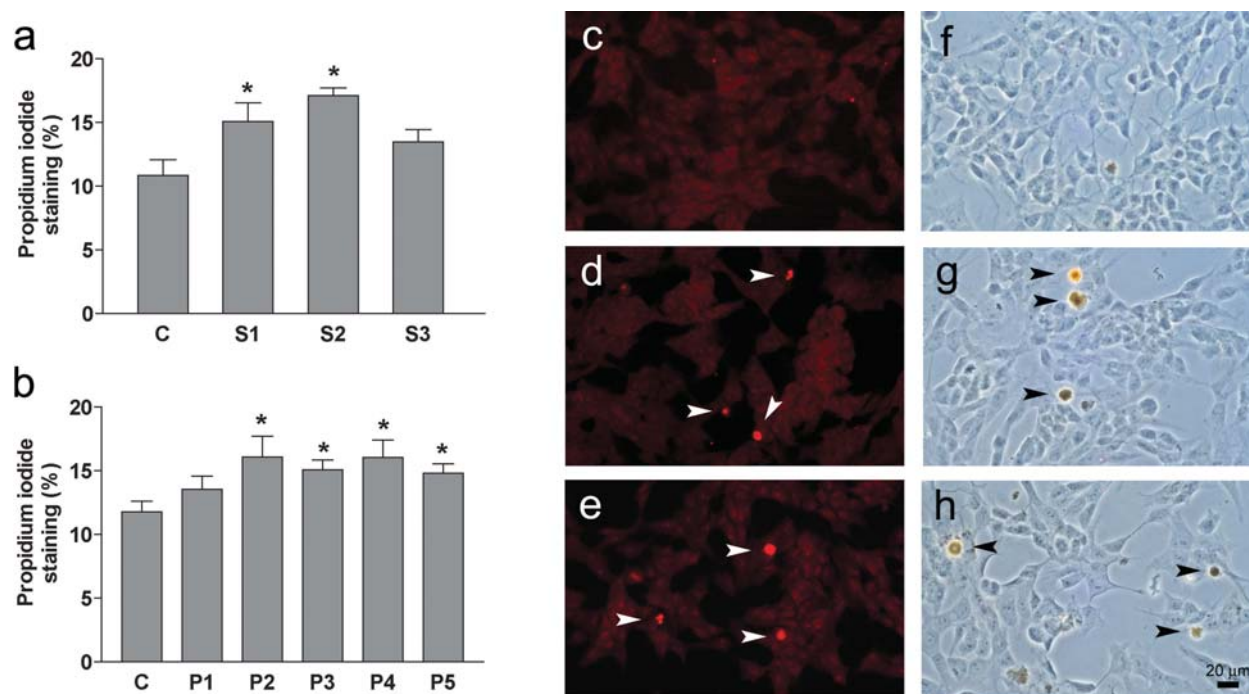
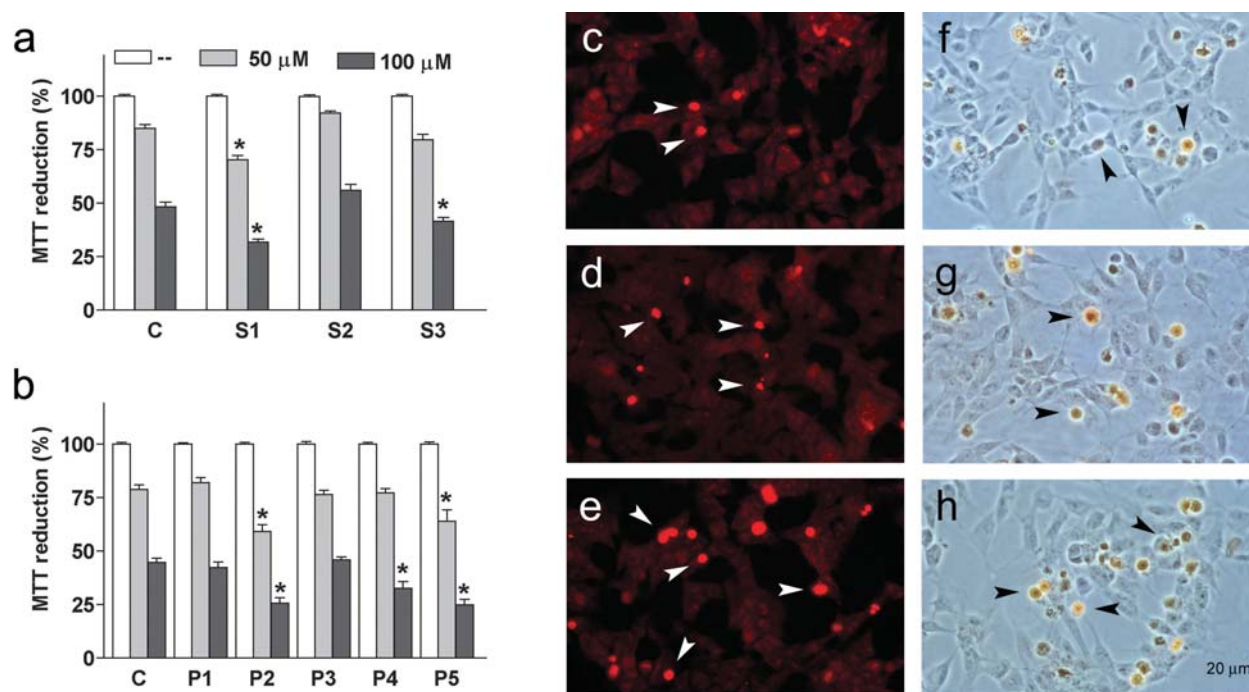


Figure 3. Basal death in SH-SY5Y neuroblastoma cells stably transfected with human *SOD1* sense or *PRDX2* antisense constructs. (a) Percentage of propidium iodide fluorescence of *SOD1*-transfected clones (S1–S3) in relation to controls (C). (b) Percentage of propidium iodide fluorescence of *PRDX2* antisense clones (P1–P5) in relation to controls (C). \* $p < 0.05$  as compared with controls; see Results for statistics. (c–e) Microphotographs of cells fixed after propidium iodide staining. Faint basal staining of living cells is detectable in control cultures (c), while highly fluorescent dead cells, indicated by arrowheads, appear both in S (d) and P (e) clones. (f–h) Microphotographs of TUNEL-processed cultures of controls (f), S (g) and P (h) clones, with several positive cells for DNA fragmentation, marked by arrowheads, in both transfected clone types. Scale bar, 20  $\mu\text{m}$ .



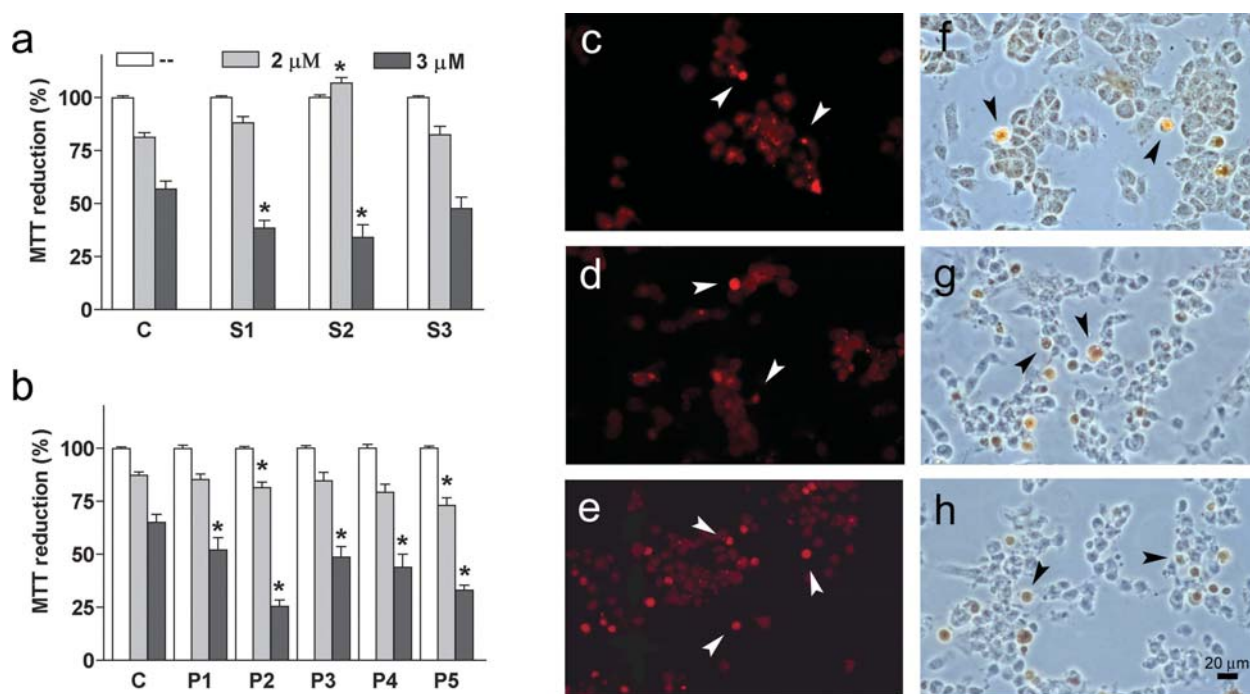


Figure 5. Effect of 24 h exposure to 2 or 3  $\mu\text{M}$  thimerosal on the viability of SH-SY5Y cells stably transfected with human *SOD1* sense or *PRDX2* antisense constructs. (a) Cytotoxicity as indicated by a decrease in the percentage of MTT reduction in *SOD1* transfected clones (S1–S3). (b) Cytotoxicity as indicated by a decrease in the percentage of MTT reduction in *PRDX2* antisense clones (P1–P5). \* $p < 0.05$  as compared with the corresponding treatment in controls (C); see Results for statistics. (c–e) Microphotographs of cells stained with propidium iodide after 24 h exposure to 2  $\mu\text{M}$  thimerosal. Some highly fluorescent dead cells are indicated by arrowheads in control cultures (c) and S clones (d), whereas a higher number of dead cells are evident in P clones (e). (f–h) Microphotographs of TUNEL-processed representative cultures of control (f), S (g) and P (h) clones after 15 h of exposure to 2  $\mu\text{M}$  thimerosal, with some positive cells indicated by arrowheads. A more disruptive effect of thimerosal is apparent on P cells. Scale bar, 20  $\mu\text{m}$ .

### Cell apoptosis under standard and oxidative stress conditions

To test whether *PRDX2* under-expression or *SOD1* over-expression increased apoptosis in transfected neuroblastoma cells under standard and oxidative conditions (see Materials and methods), four approaches were undertaken: (i) TUNEL assay; (ii) immunostaining of caspase-3; (iii) immunodetection of caspase-3 activity upon addition of a substrate (fodrin) and (iv) direct quantification of caspase-3 activity.

Under standard basal conditions, nuclear fragmentation in dead cells was more evident in either *SOD1* sense or *PRDX2* antisense clones than in controls, as detected by the TUNEL assay (fig. 3f–h). Moreover, the number of

TUNEL-positive cells increased after treatment with hydrogen peroxide (fig. 4f–h) or thimerosal (figs. 5f–h and 6). A more disruptive effect of both cytotoxic reagents on cell morphology and culture organization was evident in the *PRDX2* antisense clones (figs. 4h, 5h). ANOVA showed an effect of both factors, clonal cell type ( $F_{4,40} = 4.37$ ,  $p = 0.005$ ) and treatment ( $F_{2,40} = 39.172$ ,  $p < 0.001$ ), in the percentage of TUNEL-positive cells when comparing controls and both types of transfected cells (fig. 6).

Direct immunostaining of the 17-kDa fragment from caspase-3 cleavage revealed that cell death induced by thimerosal and hydrogen peroxide proceeded, at least partially, through caspase-3 activation, either in control or in transfected cells (data not shown).

Figure 4. Effect of 24 h exposure to 50 or 100  $\mu\text{M}$  hydrogen peroxide on the viability of SH-SY5Y stably transfected with human *SOD1* sense or *PRDX2* antisense constructs. (a) Cytotoxicity as indicated by a decrease in the percentage of MTT reduction in *SOD1* transfected clones (S1–S3). (b) Cytotoxicity as indicated by a decrease in the percentage of MTT reduction in *PRDX2* antisense clones (P1–P5). \* $p < 0.05$  as compared with the corresponding treatment in controls (C), see Results for statistics. (c–e) Microphotographs of cells stained with propidium iodide after 24 h exposure to 50  $\mu\text{M}$  hydrogen peroxide. Some highly fluorescent dead cells are indicated by arrowheads in control cultures (c), whereas a higher number of stained cells appear both in S (d) and P (e) clones. (f–h) Microphotographs of TUNEL-processed representative cultures of control (f), S (g) and P (h) clones exposed to 50  $\mu\text{M}$  hydrogen peroxide for 15 h, with positive cells indicated by arrowheads. The effect of hydrogen peroxide on cell morphology and culture disorganization is apparent mainly on P cells. Scale bar, 20  $\mu\text{m}$ .

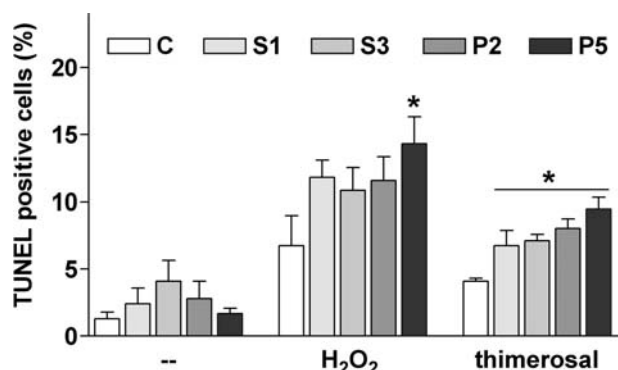
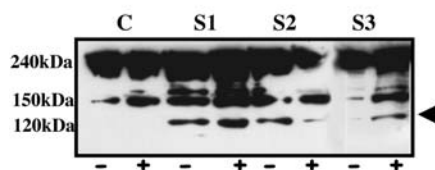


Figure 6. Percentage of TUNEL-positive cells after 15 h exposure to either 50  $\mu$ M hydrogen peroxide or 2  $\mu$ M thimerosal in SH-SY5Y control-transfected cells (C), and SH-SY5Y stably transfected with human *SOD1* sense (S) or *PRDX2* antisense (P) constructs. \* $p < 0.05$  as compared with controls (see Results for statistics).

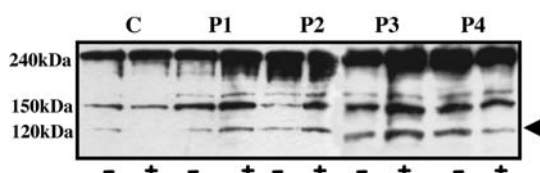
Fodrin, produced as a 240-kDa precursor protein, generates an active 150-kDa peptide which, upon activation of caspase-3, is degraded to a 120-kDa proteolytic product. Immunodetection of this 120-kDa fragment is a routine assay for cellular apoptosis. In our case, the cleaved 120-kDa fragment was barely detectable in controls (SH-SY5Y cells transfected only with the pTet-on vector) under standard conditions (lanes C in fig. 7a, b). In contrast, higher levels of cleaved fodrin were detected in protein preparations from all *SOD1* sense (fig. 7a) and *PRDX2* antisense (fig. 7b) transfected clones, pointing to an increase in apoptosis. When the immunodetection was performed after 3 h treatment with 100  $\mu$ M hydrogen peroxide, fodrin cleavage was remarkably lower in both *PRDX2* antisense and *SOD1* sense clones than in controls: (i) untransfected SH-SY5Y cells (fig. 7c, lane W) and (ii) SH-SY5Y cells transfected with the pTet-on vector (fig. 7c, lane C).

Further assessment of the immunodetection data was obtained through direct quantification of caspase-3 activity on a fluorescent substrate. The results (given as an average of all the independent clones) confirmed that, compared to controls, caspase-3 activity in both types of transfectant clones (i) was higher under standard condi-

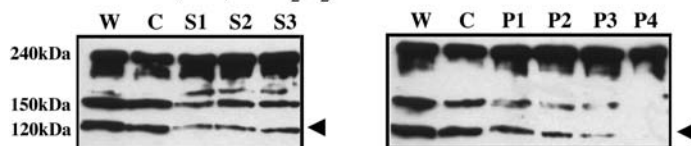
#### a. *SOD1* sense clones



#### b. *PRDX2* antisense clones



#### c. *SOD1* sense and *PRDX2* antisense clones, (100 $\mu$ M H<sub>2</sub>O<sub>2</sub>)



#### d. caspase 3 activity

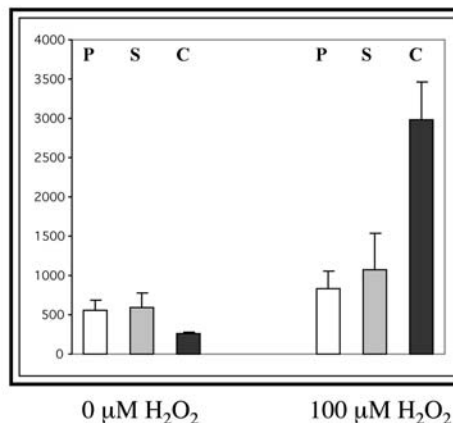


Figure 7. Substrate cleavage by caspase-3 activity. Immunodetection of fodrin in protein extracts from *SOD1* sense (a) and *PRDX2* antisense (b) transfected clones, under basal conditions (the symbols – and + indicate tetracycline induction). (c) Immunodetection of fodrin in protein extracts from tetracycline-induced *PRDX2* antisense and *SOD1* sense clones after 3 h treatment with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. (d) Fluorimetric quantification of mean caspase-3 activity in protein extracts from control cells and the stably transfected analysed clones under standard conditions (left) and after 3 h treatment with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (right). The represented values were obtained after 60 min incubation of protein extract with the substrate. The standard deviation is also indicated. Clone P5 was not available for this set of experiments. P, *PRDX2* antisense clones; S, *SOD1* sense clones, C, control pTet-on cells, W, SH-SY5Y original non-transfected cells.



tions and, (ii) increased to a much lesser extent upon addition of 100  $\mu$ M hydrogen peroxide (fig. 7d).

## Discussion

Gene dosage imbalance associated with aneuploidies may introduce multiple subtle differences in the expression of many genes, which will eventually converge on a complex phenotype, such as DS. In this context, understanding of DS pathogenesis requires complementary strategies to the systematic characterization of genes on chromosome 21. Among them, the search for DS differentially expressed genes, although not devoid of pitfalls, is a promising approach.

SSH performed on DS and control fetal brains revealed that *PRDX2* is under-expressed in DS samples. This was further confirmed and quantified by real-time PCR analyses. Three individuals of each population were used for SSH and 13 additional samples (7 DS and 6 controls) were used to validate the data by quantitative RT-PCR. In total, 19 different samples were analysed. The number of samples is not a trivial issue, as the variability inherent to the individual genetic background hinders the detection of differentially expressed genes. Therefore, statistical significance can only be attained by increasing the number of samples analysed.

*PRDX2* is exclusively expressed in large neurons, such as hippocampal pyramidal and Purkinje cells [16]. As significant neuronal loss in several brain regions, including the cerebral cortex, hippocampus and cerebellum, is associated with DS [28], *PRDX2* under-expression may be a direct consequence of the reduction in this neuronal population. Concurrently, cellular *PRDX2* expression levels might decrease as a direct effect of the altered expression of a gene or group of genes located on chromosome 21. Recently, and in agreement with our results, decreased *PRDX2* protein levels have been reported in DS fetal brains, albeit not in adult samples, after two-dimensional-protein gel electrophoresis [29, 30].

Although *PRDX2* is not located on chromosome 21, the antioxidant activity of *PRDX2* and its contribution to cell redox status support the relationship between under-expression of this gene and the reported enhanced sensitivity to oxidative stress of DS neurons [31, 32]. In this respect, previous reports have shown a direct relationship between over-expression of several *PRDX* family members and protection from the apoptosis caused by oxidative agents like hydrogen peroxide and etoposide [15, 20–22, 33–35]. Moreover, treatment with antisense *PRDX2* oligonucleotides, which effectively prevented *PRDX2* expression, increased cell sensitivity to oxidative stress caused by radiation [36].

Our antisense construct did not entirely abolish *PRDX2* expression, but decreased it to 0.6- to 0.9-fold, well within

the range of DS fetal brain expression. The under-expression of *PRDX2* slightly increased the rate of apoptosis in cultured cells under standard conditions, as deduced from our data on caspase-3 activity (by immunodetection and fluorescence of cleaved substrates) and in agreement with the increased spontaneous death detected by propidium iodide staining. Furthermore, *PRDX2* antisense clones also showed higher mortality than controls under the oxidative stress caused by hydrogen peroxide and thimerosal. However, cell death proceeded differently depending on the toxic agent. Notably, after addition of hydrogen peroxide and in contrast to treatment with thimerosal, the activation of caspase-3 was much higher in controls than in transfected cells, the latter mostly undergoing necrosis or a caspase-3 independent apoptosis.

The data available on *SOD1* over-expression are controversial. While some authors argue that over-expression deregulates the cell redox status and leads to increased oxidative damage [4], others support increased cell viability and organism longevity, at least in *Drosophila* [37]. These later effects have been mainly attributed to a concomitant glutathione peroxidase (GPX) activation, which balances *SOD1* activity [38, 39]. Here, the effect of *SOD1* over-expression on cell viability was deleterious both under standard culture conditions and under oxidative stress caused by hydrogen peroxide, which was consistent with the lack of activation of GPX enzymatic activity (our data, not shown) and in agreement with Lee et al. [40]. The partial shift to caspase-3 mediated death at 100  $\mu$ M hydrogen peroxide was lower than in the controls but similar to that observed in the *PRDX2* antisense clones. Etoposide, a well-known inhibitor of topoisomerase II, did not differentially affect transfected cells with either construct with respect to controls, implying that neither construct affected cellular DNA replication, repair or recombination pathways. However, both types of transfected cells were more sensitive than controls to hydrogen peroxide (a producer of oxygen radicals) and thimerosal (a sulphhydryl-oxidizing agent) supporting the direct involvement of *SOD1* and *PRDX2* activities in the cellular response to oxidative stress caused by hydroxyl radicals or due to protein thiol oxidation. Remarkably, *PRDX2* antisense clones were more sensitive to these two cytotoxic agents, particularly to thimerosal. Given that the active catalytic site of the dimeric enzyme involves thiol groups, thimerosal treatment may inactivate the already depleted *PRDX2* cellular pool by oxidation of these catalytic groups.

To sum up, under standard growth conditions, either *PRDX2* under-expression or *SOD1* over-expression decreased cell viability to a similar level and increased the rate of caspase-3-mediated apoptosis. The decrease in cell viability was more evident in both cell lines, but particularly in *PRDX2* antisense clones, when treated with cytotoxic oxidative agents. In this case and when com-

pared to controls, cell death proceeded mainly via necrosis or caspase-3-independent apoptosis (at least in hydrogen peroxide-treated cells).

Mental retardation in DS has often been associated with higher neuronal sensitivity to physiological oxidative radicals and increased neuronal apoptosis [4, 32, 33, 41]. Increased cellular activity of SOD1 as a result of gene-dosage imbalance was considered a major factor for the DS-associated neuronal oxidative stress. Now, according to our work, *PRDX2* is under-expressed in DS fetal brains. In this context and at the cellular level, it may be relevant that the altered expression of either gene (i) decreased cell viability under standard growth conditions and (ii) increased sensitivity under oxidative stress. The cumulative effects of the combined *PRDX2* and *SOD1* alterations, and the contribution of other antioxidant enzymes, among them the PRDX family members [29, 30], on the cellular redox state of DS neurons remain to be elucidated. In any case, our data further support the relationship between a decrease in the oxidative stress defence mechanisms and the apoptosis and neurodegeneration observed in DS brains. Finally, we also postulate that under-expression of *PRDX2* contributes to the DS-neuron oxidative alterations to a similar extent as *SOD1* over-expression.

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