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Histone deacetylase 4 promotes ubiquitin-dependent proteasomal degradation of Sp3 in SH-SY5Y cells treated with di(2-ethylhexyl) phthalate (DEHP), determining neuronal death

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ABSTRACT

Phthalates, phthalic acid esters, are widely used as plasticizers to produce polymeric materials in industrial production of plastics and daily consumable products. Animal studies have shown that di(2-ethylhexyl)phthalate (DEHP) may cause toxic effects in the rat brain. In the present study, chronic exposure to DEHP (0.1–100 μM) caused dose-dependent cell death via the activation of caspase-3 in neuroblastoma cells. Intriguingly, this harmful effect was prevented by the pan-histone deacetylase (HDAC) inhibitor trichostatin A, by the class II HDAC inhibitor MC-1568, but not by the class I HDAC inhibitor MS-275. Furthermore, DEHP reduced specificity protein 3 (Sp3) gene expression, but not Sp3 mRNA, after 24 and 48 h exposures. However, Sp3 protein reduction was prevented by pre-treatment with MC-1568, suggesting the involvement of class II HDACs in causing this effect. Then, we investigated the possible relationship between DEHP-induced neuronal death and the post-translational mechanisms responsible for the down-regulation of Sp3. Interestingly, DEHP-induced Sp3 reduction was associated to its deacetylation and polyubiquitination. Co-immunoprecipitation studies showed that Sp3 physically interacted with HDAC4 after DEHP exposure, while HDAC4 inhibition by antisense oligodeoxynucleotide reverted the DEHP-induced degradation of Sp3. Notably, Sp3 overexpression was able to counteract the detrimental effect induced by DEHP. Taken together, these results suggest that DEHP exerts its toxic effect by inducing deacetylation of Sp3 via HDAC4, and afterwards, Sp3-polyubiquitination.

Introduction

The di(2-ethylhexyl)phthalate (DEHP) is the most common commercial plasticizer used in the production of polyvinylchloride plastics, which have a variety of applications including storage of human blood, food packaging, cosmetics, and medical devices (Kavlock et al., 2002). It has been reported that at an appropriate concentration DEHP exerts toxic effects on the central nervous system following both prenatal and postnatal exposures. Indeed, prenatal exposure to DEHP disrupts brain development and decreases brain weight in rats (Tanida et al., 2009). Particularly, it has been shown that DEHP poses a negative impact on hippocampal development in male rats (Smith et al., 2011; Tanida et al., 2009). Postnatal DEHP exposure is known to cause motor hyperactivity (Masuo et al., 2004b) and significantly decreases the number of midbrain dopaminergic neurons (Masuo et al., 2004a; Tanida et al., 2009). In humans, association between phthalate exposure and autism spectrum disorders has been suggested (Testa et al., 2012), although this conclusion is limited by relatively small sample sizes (Rossignol et al., 2014).

Several lines of evidence indicate that alteration in the expression and function of histone deacetylases (HDACs) (Abel and Zukin, 2008) and specificity protein (Sp) transcription factors is involved in neurodegeneration (Qiu et al., 2006; Ravache et al., 2010; Ryu et al., 2003; Santpere et al., 2006). Four different classes of HDACs are known: class I HDACs (1–3 and 8) are constitutively nuclear proteins; class II HDACs (4–7, 9, and 10) are expressed in a cell-specific manner and shuttle between the nucleus and cytoplasm (de Ruijter et al., 2003); class III HDACs or sirtuins consist of seven members (SIRT1–SIRT7); and class IV HDACs, currently composed of one member, HDAC11, of which little is known (Haberland et al., 2009). Sp transcription factors are a family of proteins containing zinc-finger motifs that bind to G-C/

Abbreviations: DEHP, di(2-ethylhexyl)phthalate; HDAC, histone deacetylase; Sp1, Sp3 and Sp4, specificity proteins 1, 3 and 4; AS, antisense oligodeoxynucleotide; MS, missense oligodeoxynucleotide.

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T rich sequences (Suske, 1999). Sp transcription factors regulate the expression of a large number of genes, including most "housekeeping" genes (Mao et al., 2007). Interestingly, it has been shown that forced expression of Sp1 or Sp3 is neuroprotective (Ryu et al., 2003). Since it has been demonstrated that HDACs and Sp transcription factors are involved in several neurotoxic insults such as those induced by polychlorinated biphenyls (Formisano et al., 2011), brain ischemia (Formisano et al., 2013), and Huntington's disease (Ravache et al., 2010), we investigated the possible relationship between DEHP-neurotoxicity and Sp transcription factors in neuroblastoma SH-SY5Y cells.

Materials & methods

Drug and chemicals. Di(2-ethylhexyl) phthalate (DEHP) (stock solution 2.56 M) (N-11226; lot: 284400) was purchased from Chem Service (West Chester, USA). Culture media and sera were obtained from Invitrogen (Milan, Italy). The HDAC inhibitors trichostatin A (TSA) (T8552) (stock solution 1 μM), MS-275 (EPS002) (stock solution 100 μM), MC-1568 (M1824) (stock solution 1 M), and MG-132 (C2211) (stock solution 40 mM) were obtained from Sigma-Aldrich (St. Louis, MO) and pan-caspase inhibitor, z-VAD-fmk (Merck Chemicals, Nottingham, UK) (stock solution 100 mM). All chemicals were diluted in a cell culture medium. For those requiring dilution in DMSO, the final DMSO concentration was 0.1%. DMSO was added to the control cells at the maximum concentration used for the treatments. Under these conditions, DMSO did not induce cellular toxicity.

Cell lines and culture conditions. Human neuroblastoma SH-SY5Y cells (IRCCS Azienda Ospedaliera Universitaria San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), L-glutamine (2 mM), pyruvic acid (1 mM), 1× non-essential amino acids and 1× antibiotics. All experiments were conducted in cultures containing cells between the 15th and 25th passages. After 24 h of cell seeding, DEHP (0.1 μM, 10 μM, 50 μM, 100 μM) was added in DMEM medium containing 1% FBS, for 24 and 48 h. Oligodendrocyte MO3.13 progenitor cells were prepared as described previously (Boscia et al., 2012). Animal studies have been approved by the Animal Care Committee of "Federico II", University of Naples, Italy. Cortical neurons were prepared from 17-day-old Wistar rat embryos (Charles River, Calco, Milan, Italy) as previously described (Formisano et al., 2013) and used after 10 days. Neurons were grown in modified Eagle medium/F1 containing 30% glucose, 5% of deactivated fetal bovine serum and 5% horse serum, L-glutamine (2 mmol/l), penicillin (50 U/ml), and streptomycin (50 μg/ml). For all the experiments with HDAC inhibitors and pan-caspase inhibitor, after cell seeding, cells were pre-treated for 2 h with TSA (50 nM), MS-275 (5 μM) and MC-1568 (5 μM) and for 3 h z-VAD-fmk.

Cell transfection. Anti-sense and missense oligonucleotides (ODNs) specifically designed for HDAC4 (HDAC4 AS and HDAC4 MS) were transfected into SH-SY5Y cells (70% confluence) and cortical neurons (60% confluence) with Lipofectamine in Optimem medium, according to the protocol by the manufacturer (Invitrogen Srl, S. Giuliano Milanese Italy). Afterwards, Optimem was removed and a fresh medium was added for 24 and/or 48 h, alone or in combination with DEHP 50 μM. In brief, oligonucleotides (1 μM) were dissolved in Lipofectamine and then incubated at room temperature for 30 min before being added to the culture medium. Nucleotide sequences were designed to target the human HDAC4 mRNA (NM_006037.3): HDAC4 AS and HDAC4 MS are GCCCACGCCCCACCAGCAGGC (7317–7337) and GCCCACGCCCCATT GGCAGGC. ODN target rat HDAC4 mRNA were already published (XM_343629.3) (Matsuda et al., 2011). pN3 and pN3–Sp3, the plasmid carrying Sp3 cDNA, were kindly provided by Prof. G. Suske (Marburg, Germany). Transient transfection of 1 μg DNA in 12-multiwell plates was performed using Lipofectamine in Optimem, for 6 h in SH-SY5Y cells and for 2 h in cortical neurons (1×10^5 cells/well). Finally, cells were exposed to 50 μM DEHP for 48 h and subjected to Annexin V. For Western blot analysis, cells were plated in 100 mm dishes, and transiently transfected with 15 μg of DNA using Lipofectamine in Optimem, for 6 h in SH-SY5Y cells and for 2 h in cortical neurons. After all, cells were exposed to 50 μM DEHP for 48 h. To evaluate cell transfection efficiency, constructs and ODNs were mixed with a plasmid encoding the enhanced green fluorescent protein marker. Transfection efficiency was ≅80% for SH-SY5Y cells and ≅70% for cortical neurons (data not shown).

Determination of cell viability. Cell viability was determined using the dimethylthiazolyl-2-5-diphenyltetrazolium-bromide (MTT, Sigma) staining, at 24 and 48 h after DEHP treatments in SH-SY5Y cells and oligodendrocyte MO3.13 progenitor cells at concentrations of 0.1 μM, 10 μM, 50 μM and 100 μM and in cortical neurons at concentrations of 0.01 μM, 0.1 μM, 10 μM, 50 μM, and 100 μM.

For the MTT with HDAC inhibitors and z-VAD-fmk, SH-SY5Y cells were first seeded into 12 well-plates, treated with 0.1% DMSO (CTL), and pre-treated for 2 h with TSA, MS-275, and MC-1568 and for 3 h with z-VAD-fmk. Cells were exposed to DEHP (50 μM) for 48 h. After treatment, cells were incubated with 0.5 mg/ml MTT for 1 h at 37 °C. Afterwards, the supernatant was discarded, the insoluble formazan precipitates were dissolved in 0.5 ml DMSO, and the absorbance was detected at 540 nm. Each experiment was performed in triplicate. Values from each treatment were reported as a percentage of the relative control.

Annexin V staining. SH-SY5Y cells (1×10^5 cells/well) were seeded into each well of a 12-well plate and pre-treated with for 2 h with HDAC inhibitors or for 6 h with z-VAD-fmk. In the experiments of Sp3 overexpression and HDAC4 knock-down, SH-SY5Y (1×10^5 cells/well) and cortical neurons (1×10^5 cells/well) were transfected with pN3–Sp3 plasmid carrying Sp3 cDNA and with the empty vector pN3 or with HDAC4 AS and HDAC4 MS. For apoptosis staining experiments, cells were exposed to DEHP (50 μM) for 48 h. 5×10^5 cells were harvested and stained according to the Tali Apoptosis Kit instructions (Invitrogen, Molecular Probes, Life Technologies). Briefly, cells were incubated with Annexin V Alexa Fluor 488 for 20 min at room temperature and then deposed in suspension in a slide. Fluorescence was scanned with the Tali Image based Cytometer (Invitrogen, Life Technologies). Apoptotic cells were Annexin V positive and the negative sample was acquired for control staining. The cells were analyzed using a Tali™ Image byuring captures 10 images of a stained sample, automatically analyzes the images using digital image-based cell counting and fluorescence-detection algorithms, and displays an accurate quantitative analysis of live, dead, and apoptotic cell populations. All measurements were performed in triplicate.

Western blot analysis. Cells were lysed in buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitors. They were incubated for 1 h on ice and centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatants (100 μg for Sp1, 3, 4, pro-caspase, cleaved caspase 3 and 50 μg for Acetyl-H3 and Acetyl-tubulin) were incubated with the following buffer: 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. The extracts were electrophoreted through SDS-8% polyacrylamide gel for the detection of Sp1, 3, 4, pro-caspase and cleaved caspase 3, and through SDS-12% polyacrylamide gel for Acetyl-H3 and Acetyl-tubulin. The gels were electroblotted onto polyvinylidene difluoride membrane (Amersham Biosciences) in transfer buffer (48 mM Tris–HCl, 39 mM glycine, and 20% methanol). Membranes were then treated with a blocking solution for 2 h at room temperature (RT) (10% dry milk and 0.1% Tween 20 in Tris-buffered saline) and incubated overnight at 4 °C

with the following specific antibodies: anti-pro-caspase-3 (1:200, sc-7272, monoclonal antibody, Santa Cruz Biotechnology Inc.), anti-cleaved caspase-3 (1:1000, cat: AAP-113, polyclonal antibody, Stressgen Biotechnologies Corporation), anti-Sp1 (1:1000, cat: sc-14027, polyclonal antibody, Santa Cruz Biotechnology Inc.), anti-Sp4 (1:1000, cat: sc-645, polyclonal antibody, Santa Cruz Biotechnology Inc.), anti-Sp3 (1:500, sc-644, polyclonal antibody, Santa Cruz Biotechnology Inc.), anti Acetyl-H3 (1:1000, cat: 06-599, rabbit polyclonal antibody, Millipore corporation), anti-acetyl-tubulin (1:10,000, T7451, mouse monoclonal antibody, Sigma-Aldrich), anti-tubulin (1:1000, cat: sc-654, polyclonal antibody, Santa Cruz Biotechnology Inc.), anti-HDAC4 (1:1000, cat: sc-11418, polyclonal antibody, Santa Cruz Biotechnology Inc.) and anti-β-actin (1:2000, A3853, monoclonal antibody, Sigma-Aldrich, St. Louis, MO). For Sp3, three different Sp3 isoforms of almost 115, 70 and 68 kDa have been detected; we decided to analyze only the 115-kDa isoform, since it is biologically active (Ammanamanchi and Brattain, 2001). Immunoreactive bands were detected using an enhanced chemiluminescence reagent (GE Healthcare), and analyzed by the Chemi-Doc Imaging System (Bio-Rad Laboratories, Hercules, CA). The experiments were replicated three times.

Chromatin immunoprecipitation (ChIP) assay and quantitative real-time-PCR. Cells plated in 100-mm dishes (5×10^6 cells per dish), and treated with DEHP (50 μM) alone or after pretreatment 2 h with MC-1568 were cross-linked with 1% formaldehyde for 10 min at room temperature, crosslinking was stopped by incubating cells with glycine (0.125 M) for 5 min at room temperature. Cells were washed twice with cold PBS containing protease inhibitors, harvested, and centrifuged at 4000 rpm at 4 °C for 3 min. The cellular pellet was lysed in buffer containing 50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, and protease inhibitors. After that, to keep cell samples at a low temperature, it was fragmented by sonication into 200–500 bp fragments with the Bioruptor instrument (Diagenode) for 20 min at high potency in a tank filled with ice/water. Samples were centrifuged at 14,000 rpm for 15 min at 4 °C and, then, diluted in IP buffer (16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, 0.01% SDS, protease inhibitors). A fraction of the sample was conserved as an input. The sample was shaken gently at 4 °C for 2 h with protein A (Protein A Agarose/Salmon Sperm DNA, 16-157C, Upstate). After centrifugation at 2000 rpm at 4 °C for 1 min, the supernatant was immunoprecipitated overnight at 4 °C with a 10 μl anti-Sp3 antibody (sc-644, Santa Cruz Biotechnology Inc.). IgG rabbit antibody was used as a negative control. After incubation with protein A (Protein A Agarose/Salmon Sperm DNA, 16-157C, Upstate) at 4 °C for 4 h, beads were sequentially washed with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8.1) and twice with Tris/EDTA buffer (10 mM Tris-HCl, pH 8.1 and 1 mM EDTA). The precipitates were extracted twice using immunoprecipitation elution buffer (1% SDS, 0.1 M $NaHCO_3$) and NaCl (5 M) was added to the total eluates and incubated at 65 °C for 6 h. DNA fragments were purified by phenol–chloroform extraction and ethanol precipitation, and dissolved in sterile water. DNA samples were then analyzed for real-time PCR using Fast SYBR Green Master Mix (Applied Biosystems). Data obtained from three different experiments of real time PCR were expressed as a percentage of the relevant controls, all normalized for the DNA input. IgG controls were performed (not shown), and the values were subtracted from results for ChIP samples. The oligonucleotides used for the amplification of immunoprecipitated DNA of cyclooxygenase-2 (COX-2) and Neuroglobin (Ngb) promoter regions were already been published (Xu and Shu, 2007; Zhang et al., 2011). The experiments were replicated three times.

Reverse transcription-real time PCR. Total RNA was isolated according to the supplier's protocol (TRI Reagent; Sigma-Aldrich, St. Louis, MO) and treated with DNAse I (1 U/μl) for 30 min at RT. The first strand cDNA synthesis was achieved with SuperScript reverse transcriptase (Invitrogen S. Giuliano Milanese, Italy). Real-time quantitative PCR was performed with the fluorescent dye SYBR Green methodology, using the FAST SYBR Green Master Mix (Applied Biosystem) and performed in the ABI Prism 7700 (Applied Biosystem). Primer pairs for target genes were the following: for Sp1 Forward (Fw) 5′-TTGAAAAAGG AGTTGGTGGC-3′, and Reverse (Rv) 5′-TGCTGGTTCTGTAAGTTGGG-3′ (NM_001251825.1), Sp3 Fw 5′-CCAGGATGTGGTAAAGTCTA-3′, Rv 5′-CTCCATTGTCTCATTTCCAG-3′ (NM_001172712.1), Sp4 Fw 5′-GCGGAA AAAGAGGCAGAGCC-3′, Rv 5′-CTTCTGTAGCCATCGCCGC-3′ (NM_003112.3), HDAC4 Fw 5′-CAGATGGACTTTCTGGCC-3′, Rv 5′-CTTGAGCT GCTGCAGCTTC-3′ (NM_006037.3), HDAC5 Fw 5′-TGGCCTCGGATGGG CATTAG-3′, Rv 5′-CATCCCATCTGCCGACTCGT-3′ (NM_001015053.1), HDAC6 Fw 5′-ACCTAGGTTAGCTGAGCGGA-3′, Rv 5′-TGCTCGAGGCTA GAGGAGTT-3′ (NM_006044.2), HDAC7 Fw 5′-CTCGGAGGCTGACAGTGA CC-3′, Rv 5′-GGTCCAGGAGGAGAATGGGC-3′ (NM_001098416.2), HDAC9 Fw 5′-AGCAACCAGGCAGTCACCTT-3′, Rv 5′-GCCTGCTCCCCAGA TTCCAT-3′ (NM_001204148.1), HDAC10 Fw 5′-TTGACTCAGCCATCGG GGAC-3′, Rv 5′-TAGGGCACTCTGACATGGCG-3′ (NM_032019.5), ribosomal protein L19 (L19) Fw 5′-CTAGTGTCCTCCGCTGTGG-3′, Rv 5′-AAGGTGTTTTTCCGGCATC-3′ (NM_032019.5), Ngb Zhang et al., 2011, COX-2 (Alique et al., 2007) and HDAC class I (Kawabata et al., 2010). Samples were amplified simultaneously in triplicate in one assay run, and the threshold cycle (Ct) value for each experimental group was determined. Normalization of data was performed using L19. To evaluate differences in mRNA content among groups, normalized values were entered into the formula $2^{-\Delta\Delta Ct}$. All data were normalized to control "housekeeping genes", known to be stable after DEHP treatment, actin, and HPRT (hypoxanthine phosphoribosyl transferase). Although none of these genes showed a statistically significant change following DEHP exposure, the gene which exhibited the least variability was L19 (not illustrated). The experiments were replicated four times.

Immunoprecipitation. Cells were seeded into a 100-mm tissue culture and grown to approximately 80% confluence. Cells were treated with DEHP (50 μM), alone or pre-treated for 2 h with MC-1568 (5 μM), and transiently transfected with HDAC4 AS (10 μM). Total cell extracts for each group were resuspended using immunoprecipitation lysis buffer (50 mM Tris–HCL, pH 7.5, 1, Triton 1%, β-glicerol 10 mM, NaF 100 mM, Na_3VO_4 100 mM, 150 mM NaCl, and 1 mM EDTA) with the addition of protease inhibitor cocktail (1/100) P-8140 (Sigma). Aliquots of 1500 μg were used for the experiments in a final volume of 1 ml, followed by the addition of 20 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). The reactions were placed on a rotator at 4 °C for 4 h. A 900 μl aliquot of supernatant was used for the immunoprecipitation. Rabbit polyclonal anti-Sp3 (3 μg) (sc-644) HDAC3 (3 μg) (sc-11417) or normal rabbit IgG (3 μg) was added to either treatment set, followed by the addition of 20 μl of protein A/G PLUS-agarose beads. The samples were then placed on a rocker at 4 °C over-night. The supernatants were removed, and washed two times with 500 μl of cold PBS 1× plus protease inhibitors. The pellets were resuspended in 20 μl of loading buffer, boiled for 10 min and centrifuged. The immunoprecipitated supernatant was separated by SDS-10% and blotted onto polyvinylidene difluoride membrane (Amersham Biosciences) in transfer buffer (48 mM Tris–HCl, 39 mM glycine, and 20% methanol). Membranes were then treated with a blocking solution for 2 h at RT (10% dry milk and 0.1% Tween 20 in Tris-buffered saline) and incubated overnight at 4° with anti-ubiquitin (1:1000, cat: sc-8017, mouse monoclonal antibody, Santa Cruz Biotechnology Inc.), anti-acetyl-lysine (1:1000, cat: 9441,

polyclonal antibody, Cell Signaling), anti-Sp3 (1:1000, cat: sc-644, rabbit polyclonal antibody, Santa Cruz Biotechnology Inc.), and anti-HDAC4 (1:1000, cat: sc-11418, polyclonal antibody, Santa Cruz Biotechnology Inc.). Immunoreactive bands were detected using an enhanced chemiluminescence reagent (GE Healthcare), and analyzed by the Chemi-Doc Imaging System (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Data are expressed as mean ± S.E.M. Statistical comparisons among the experimental groups were performed using one way ANOVA followed by the Newman–Keuls test. P value < 0.05 was considered statistically significant.

Results

DEHP-induced apoptotic cell death is inhibited by class II HDAC inhibitor MC-1568 in SH-SY5Y cells

When SH-SY5Y cells were exposed to different concentrations of DEHP (0.1–100 μM) for 24 and 48 h, a dose-related reduction of cell viability was found by MTT assay (Figs. 1A,B). 50 μM DEHP exposure resulted in damage to approximately 50% of cells, and was therefore chosen for all experiments, unless indicated otherwise. Notably, DEHP (0.1–100 μM) did not induce toxicity in oligodendrocyte MO3.13 progenitor cells at 24 and 48 h (Supplementary Figs. 1A,B).

Treatment with pan-HDAC inhibitor trichostatin A (TSA; 50 nM) (Formisano et al., 2013) and class II HDAC inhibitor MC-1568 (5 μM) (Nebbioso et al., 2010; Spallotta et al., 2013), but not class I HDAC inhibitor MS-275 (5 μM) (Marinova et al., 2011), resulted in a significant improvement in cell viability, compared to cultures exposed to DEHP alone. Treatment with HDAC inhibitors alone had no effect on cell viability (Fig. 1C).

The HDAC specificity of TSA, MS-275, and MC-1568 was validated by measuring their effects on the levels of acetylated forms of histone H3 (indicating activity of class I and II HDACs) and tubulin (indicating activity of class II HDACs) (Galmozzi et al., 2013). Results showed that TSA and MC1568 increased the acetylation of histone H3 and tubulin, whereas MS-275 increased the acetylation of histone H3, but not of tubulin, compared to the controls (Supplementary Fig. 2A). Furthermore, SH-SY5Y cells exposed to DEHP showed a dose-related increase of active caspase-3, that was absent in control cells (Fig. 1D). This increase was completely inhibited by pre-treatment with MC1568 (Fig. 1E). Furthermore, apoptosis was quantified using FITC-conjugated Annexin V

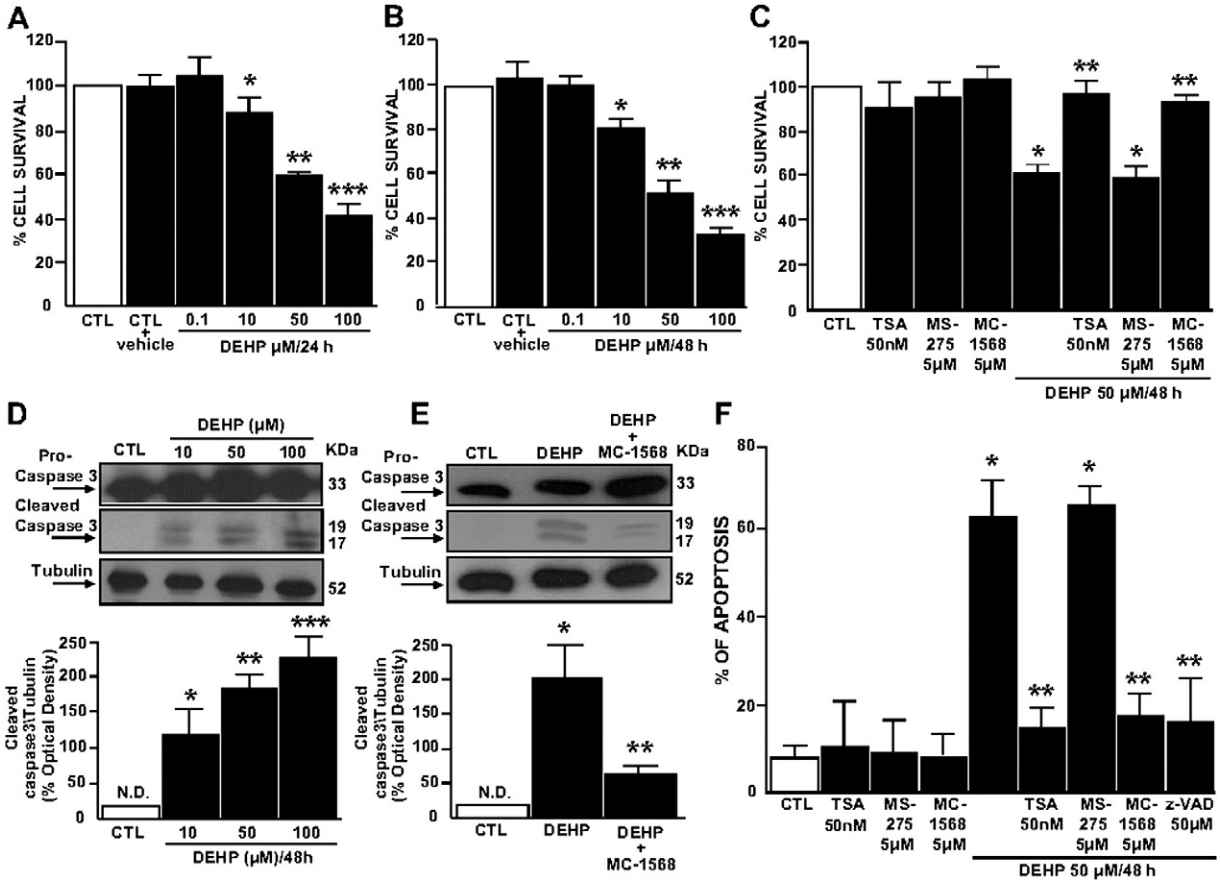

Fig. 1. Effect of DEHP on SH-SY5Y cell survival. A,B) Effect of 24 and 48 h DEHP (0.1, 10, 50 and 100 μM) exposure on the mitochondrial activity. Bars represent mean ± S.E.M. obtained in four independent experimental sessions. *P < 0.05 vs. control (CTL), CTL + vehicle, and 0.1 μM DEHP; **P < 0.05 vs. 10 μM DEHP; ***P < 0.05 vs. 50 μM DEHP. C) Effect of 50 μM DEHP at 48 h alone or with 50 nM trichostatin A (TSA), 5 μM MS-275, and 5 μM MC-1568 on the mitochondrial activity. Bars represent mean ± S.E.M. obtained in five independent experimental sessions. Cells were pre-treated with TSA, MS-275, and MC-1568 for 2 h before DEHP addition. *P < 0.05 vs. CTL; **P < 0.05 vs. DEHP. D) Western blots of pro-caspase-3, cleaved caspase-3, and tubulin levels in control conditions and 48 h after 10, 50, and 100 μM DEHP exposure. Graphs show the quantification of the ratio of cleaved caspase-3 to tubulin. Bars represent mean ± S.E.M. obtained from three independent experimental sessions. *P < 0.05 vs. CTL; **P < 0.05 vs. 10 μM DEHP; ***P < 0.05 vs. 50 μM DEHP; N.D., not detectable. E) Western blots of pro-caspase-3, cleaved caspase-3, and tubulin levels in CTL conditions or with 48 h 50 μM DEHP alone or with MC-1568. Graphs show quantification of the ratio of cleaved caspase-3 to tubulin. Bars represent mean ± S.E.M. obtained from four independent experimental sessions. *P < 0.05 vs. CTL; **P < 0.05 vs. DEHP; N.D., not detectable. F) Effect of 50 μM DEHP at 48 h alone or with 50 nM trichostatin A (TSA), 5 μM MS-275, 5 μM MC-1568 and z-VAD-fmk 50 μM on apoptosis determined using the Annexin V-Alex Fluor 488 Apoptosis kit. Bars represent mean ± S.E.M. obtained in three independent experimental sessions. Cells were pre-treated with TSA, MS-275, and MC-1568 for 2 h and 3 h with z-VAD-fmk before DEHP addition. *P < 0.05 vs. CTL; **P < 0.05 vs. DEHP.

and analyzed with a Tali™ Image Cytometer. In SH-SY5Y cells, 63% of cells were positive for Annexin V following 48 h of treatment with DEHP (50 μM), compared with only 8% of cells in the control group. Interestingly, TSA, MC-1568 and z-VAD-fmk, but not MS-275 completely reverted DEHP-induced apoptosis (Fig. 1F).

Effect of DEHP exposure on Sp1, Sp3 and Sp4 expression in SH-SY5Y cells

To verify the involvement of pro-survival transcription factors Sp1, Sp3, and Sp4 in DEHP-induced cell death, we evaluated Sp1, Sp3 and Sp4 protein levels by Western blot. It was found that DEHP (50 μM) significantly decreased the levels of Sp3, but not Sp1 and Sp4, at 24 and 48 h compared to controls (Figs. 2A–C). qRT-PCR analysis revealed that Sp1, Sp3 and Sp4 mRNAs were not significantly modified, compared to the control, 24 and 48 h after DEHP treatment (Figs. 2D–F).

Effect of proteasomal inhibitor MG-132 and class II HDAC inhibitor MC-1568 on DEHP-induced reduction of Sp3 in SH-SY5Y cells

Immunoprecipitation with a polyclonal anti-Sp3 antibody, followed by immunoblotting with a monoclonal antibody against acetyl lysine or ubiquitin, revealed that Sp3 was deacetylated and ubiquitinated after 24 h treatment with 50 μM DEHP (Figs. 3A,B). DEHP-induced deacetylation and ubiquitination of Sp3 was prevented when cells were pre-treated with MC-1568 (5 μM; Figs. 3A,B). These results indicate that DEHP induced Sp3 deacetylation and subsequently its degradation via ubiquitination. Indeed, 2 h pre-treatment of SH-SY5Y cells with MC-1568 (5 μM) or MG-132 (1 μM) was able to both significantly increase Sp3 (Figs. 3C,E) and prevent its protein reduction induced by 48 h treatment with DEHP (Figs. 3D,F). To further verify the functional consequence of Sp3 inhibition induced by DEHP, alone or after 2 h pre-treatment with MC-1568 (5 μM), ChIP with Sp3 antibody and qRT-PCR analysis for two known Sp3 target genes, COX-2 (Xu and Shu, 2007) and Ngb (Zhang et al., 2011), both constitutively expressed in SH-SY5Y cells (Alique et al., 2007; Fordel et al., 2006), was conducted.

MC-1568 and MG-132 increased Sp3 binding on COX-2 and Ngb promoter sequence (Figs. 3G,I), in parallel with an increase of COX-2 and Ngb mRNAs compared to the control group (Figs. 3M,O). As shown in Figs. 3H,L, at 24 h DEHP induced a decrease of Sp3 binding on COX-2 and Ngb genes compared to the control group and this effect was reverted by the pre-treatment with MC1568. Particularly, MC1568 reverted DEHP-induced COX-2 and Ngb mRNA decrease (Figs. 3N,P).

Effect of HDAC4 knock-down and Sp3 overexpression on cell survival in DEHP-treated SH-SY5Y cells and cortical neurons

When cortical neurons were exposed to different concentrations of DEHP (0.1–100 μM) for 24 and 48 h, a dose-related reduction of cell viability was found by MTT assay (Supplementary Figs. 3A,B). As was found in SH-SY5Y cells, 50 μM DEHP exposure resulted in damage to approximately 50% of cells, and was therefore chosen for all experiments in cortical neurons.

To evaluate the role of Sp3 in DEHP-induced toxicity, Sp3 was overexpressed in cortical neurons and in SH-SY5Y cells. As shown in Figs. 4A,D, the overexpression of Sp3 through cDNA transfection reverted DEHP-induced Sp3 reduction. Notably, Sp3 overexpression did not modify DEHP-induced HDAC4 increase, in both SH-SY5Y cells and cortical neurons (Figs. 4B,E). Interestingly, Sp3 cDNA and ODN antisense (AS) HDAC4 transfection were able to significantly reduce the number of Annexin V positive cells following 48 h DEHP exposure in SH-SY5Y cells and cortical neurons, whereas the empty vector and missense ODN (MS) controls did not (Figs. 4C,F).

DEHP exposure determines the association of HDAC4 with Sp3 in SH-SY5Y cells

To further investigate the molecular link between class II HDACs and Sp3 in DEHP-treated SH-SY5Y cells, qRT-PCR was performed with

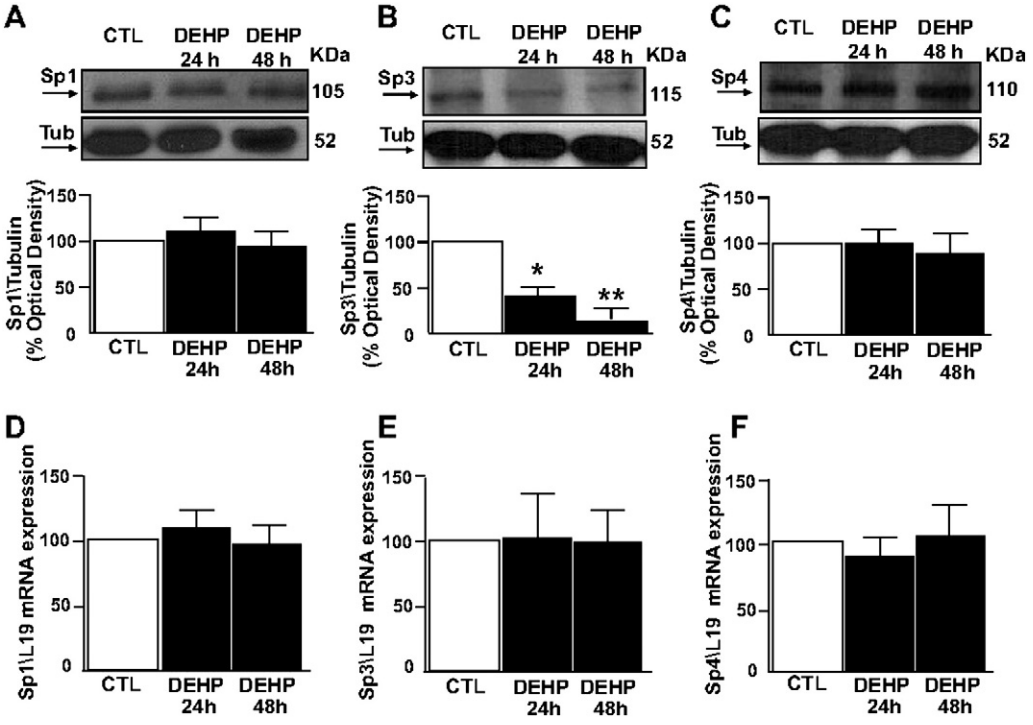

Fig. 2. DEHP-induced Sp3 protein reduction in SH-SY5Y cells. A–C) Western blots of Sp1, Sp3, and Sp4 protein levels in control conditions and 50 μM DEHP at 24 and 48 h. Graphs show the quantification of the ratio of Sp1, Sp3, and Sp4 to tubulin. Bars represent mean ± S.E.M. obtained from four independent experimental sessions. *P < 0.05 vs. CTL; **P < 0.05 vs. all. D–F) Effect of 24 and 48 h DEHP (50 μM) exposure on Sp1, Sp3, and Sp4 mRNA expression detected by RT-PCR. Bars represent mean ± S.E.M. obtained from four independent experimental sessions.

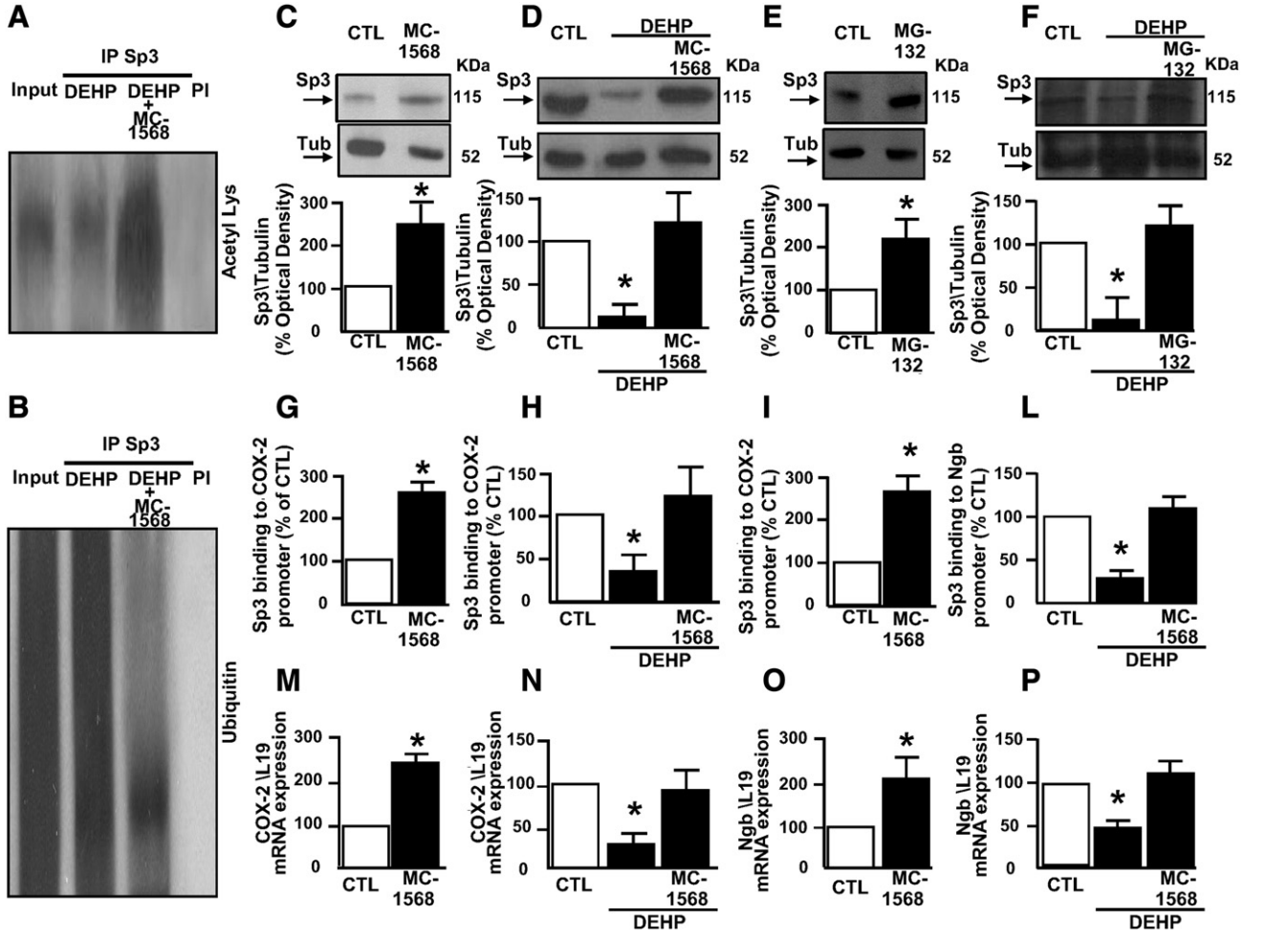

Fig. 3. DEHP-induced Sp3 protein reduction is associated with its deacetylation and poly-ubiquitination in SH-SY5Y cells. A) Representative Western blot showing immunoprecipitation between Sp3 and acetyl-lysine after 24 h 50 μM DEHP treatment alone or after 2 h pre-treatment with MC-1568. B) Representative Western blot showing immunoprecipitation between Sp3 and poly-ubiquitin after 24 h 50 μM DEHP treatment alone or after 2 h pre-treatment with MC-1568. C–F) Representative Western blots of Sp3 protein levels in control conditions (CTL), after 2 h pre-treatment with MC-1568 and MG-132 (C,E), after DEHP exposure alone or with 2 h pre-treatment with MC-1568 and MG-132, respectively (D,F). Graphs show the quantification of the ratio of Sp3 to tubulin. Bars represent mean ± S.E.M. obtained from four independent experimental sessions. *$P < 0.05$ vs. CTL. G–L) Quantification by ChIP assay of Sp3 binding on COX-2 and Ngb promoter in control conditions (CTL), after 2 h pre-treatment with MC-1568 (G,I), after DEHP exposure alone or with 2 h pre-treatment with MC-1568 and MG-132, respectively (H,L). M–P) Quantification by real time RT-PCR on COX-2 and Ngb mRNA expression in control conditions (CTL), after 2 h pre-treatment with MC-1568 (M,O), after DEHP exposure alone or with 2 h pre-treatment with MC-1568 and MG-132, respectively (N,P). Bars represent mean ± S.E.M. obtained from three independent experimental sessions for ChIP and RT-PCR experiments. *$P < 0.05$ vs. CTL.

specific primers for class I and II HDACs basally expressed in this cell line (Hornan et al., 2007; Oehme et al., 2013; Subramanian et al., 2011; Sun et al., 2013). DEHP induced a significant increase in HDAC4 at 24 and 48 h and a significant reduction of HDAC5 at 48 h, while HDAC class I and HDACs 6, 7 and 10 were not significantly modified (Figs. 5A,B). Interestingly, the HDAC4 mRNA increase was in parallel with the HDAC4 protein (Figs. 5B,C). Immunoprecipitation results showed that HDAC4 was detected in the Sp3 immunocomplex (Fig. 5D). In contrast, DEHP did not determine any interaction between HDAC3 with Sp3 and HDAC4 (Fig. 5E).

Cells transfected with ODN antisense (AS) HDAC4 for 48 h effectively showed a 70% reduction of HDAC4 expression, as compared with missense controls (MS; Fig. 5F). Interestingly, AS HDAC4 significantly increased Sp3 and reverted the DEHP-induced Sp3 protein decrease both as compared with MS (Fig. 5G). More importantly, Sp3 immunoprecipitation followed by Western blot, in cells transfected with AS HDAC4 and treated with DEHP, revealed an increase in Sp3 acetylation, in parallel with a reduction in Sp3 ubiquitination, compared with cells transfected with MS (Figs. 5H,I).

Discussion

The present study showed that exposure to DEHP induces a dose-dependent reduction of cell viability in SH-SY5Y neuroblastoma cells and in cortical neurons. Interestingly, this effect seems to be rather specific for neuronal cells since DEHP did not exert any toxic effect on oligodendrocytes. DEHP-induced cell viability reduction in SH-SY5Y cells was accompanied by an increase of cleaved caspase-3, suggesting that cell death is due to the activation of an apoptotic pathway. Furthermore, DEHP was able to reduce protein expression of the transcription factor Sp3. A change in Sp3 expression may be involved in apoptotic mechanisms, as was demonstrated by the overexpression of Sp3 preventing DEHP-induced cell death in SH-SY5Y cells and in cortical neuron cultures. To our knowledge, this is the first evidence showing Sp3 involvement in the neurodetrimental effect of DEHP. These results are in line with the known Sp3 role which favors neuronal survival. In fact, a study has shown that Sp1 and Sp3 are anti-apoptotic transcription factors in cortical neurons during oxidative stress (Ryu et al., 2003) and participate with Sp4 in the modulation of neurotoxicity resulting from

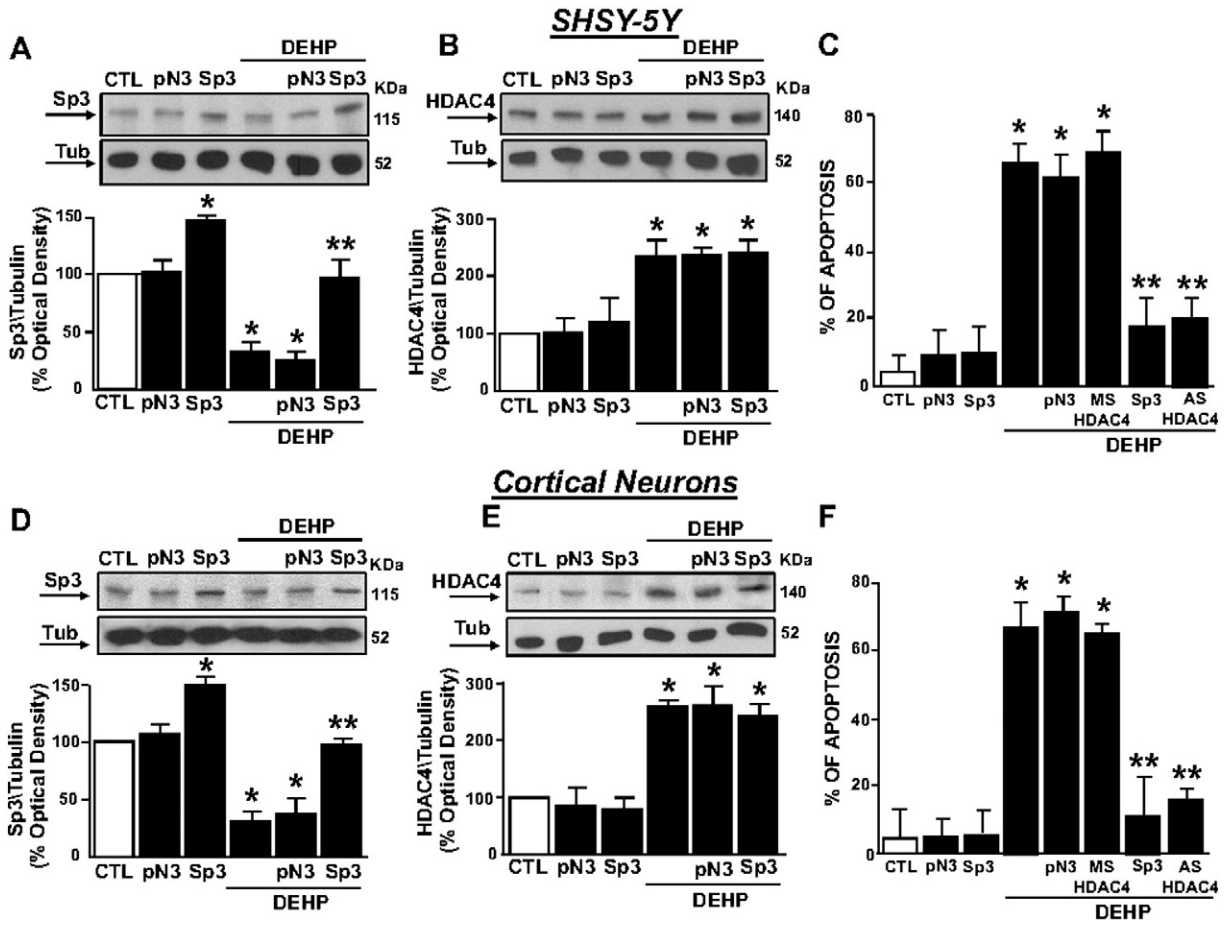

Fig. 4. Effect of Sp3 overexpression and HDAC4 knock-down on DEHP-induced Sp3 and HDAC 4 increase and on neuronal death in SH-SY5Y cells and cortical neurons. A,B,D,E) Representative Western blot showing the expression of Sp3 and HDAC4 in SH-SY5Y cells (A,B) and in cortical neurons (D,E) transfected with empty vector pN3 and with pN3 carrying Sp3 cDNA, with or without 48 h DEHP (50 μM) exposure. C,F) Effect of DEHP in SH-SY5Y cells (C) and in cortical neurons (F) transfected with empty vector pN3, pN3 carrying Sp3 cDNA, HDAC4 MS and HDAC4 AS with or without 48 h exposure DEHP (50 μM) on apoptosis determined using the Annexin Alexa Fluor 488 kit. Bars represent mean ± S.E.M. obtained in four independent experimental sessions. *P < 0.05 vs. CTL, **P < 0.05 vs. DEHP.

stroke (Mao et al., 2007). Thus, the novelty of our findings consists in the exclusive participation of Sp3, without Sp1 and Sp4, in preserving neurons from DEHP-induced neurotoxicity.

Regarding DEHP concentrations tested in the present study, they were already used by other authors in a mouse neuroblastoma cell line, with an IC50 approximately of 10 and 100 μM, respectively (Aung et al., 2014; Lin et al., 2011). Importantly, the minimal risk level of DEHP is 0.1 mg/kg/day, as reported by the Agency for Toxic substances and Disease Registry (ATSDR) (Heudorf et al., 2007). This dose corresponds in the mouse brain at a concentration of 7.8 μM (Wu et al., 2013). It should be underlined that hospital patients undergoing extracorporeal membrane oxygenation (ECMO), mechanical ventilation, total parenteral nutrition (TPN) and multiple transfusions can be exposed to high DEHP levels, resulting in exposures between 0.005 and 8.5 mg/kg bw/day (Cooper et al., 2008). These levels of exposure correspond in the mouse brain to a concentration range (0.4–640 μM) comparable to the concentrations used in the present study. Therefore the doses of DEHP used in this study are relevant to the environmental level of DEHP exposed to humans. Furthermore, DEHP can penetrate the blood–brain barrier (BBB); Wu et al. (2013) found DEHP in the brain homogenates after the administration of 0.1, 1 or 10 mg/kg thus demonstrating the possibility of DEHP to cross the blood–brain barrier. In addition, BBB permeability increases following traumatic, ischemic (Fernández-López et al., 2012) or infectious brain insults, and it may last from several days to weeks and even years after the acute event (Korn et al., 2005; Strbian et al., 2008). Moreover, we identified a possible mechanism by which Sp3 protein levels were reduced by DEHP in neuroblastoma cells. DEHP neurotoxicity and the reduction in Sp3 protein levels were prevented by the class II HDAC inhibitor MC-1568, suggesting the involvement of class II HDACs in DEHP-induced Sp3 reduction. Interestingly, the reduction of Sp3 levels is due to its deacetylation and subsequent ubiquitination, a mechanism in which HDAC4 seems to be involved. In fact, DEHP exposure did not induce a change in Sp3 mRNA, whereas it increased Sp3 protein deacetylation coupled to its ubiquitination. In addition, Sp3 reduction was reverted by both the proteasome inhibitor MG-132 and MC-1568. HDAC involvement in mechanisms leading to cell toxicity via Sp3 reduction is further demonstrated by MC-1568 being able to significantly prevent DEHP-induced neurotoxicity. Interestingly, also HDAC4 knock-down was able to counteract the neurodetrimental effect of DEHP, in SH-SY5Y neuroblastoma cells and cortical neurons. Our results are in accordance with previous reports demonstrating that Sp3 acetylation acts as a switch that controls the repression and activation role of this transcription factor (Ammanamanchi et al., 2003). It seems that the process of Sp3 deacetylation involves HDAC4, since mRNA and protein are increased by DEHP and specific inhibition of this enzyme, obtained by ODN (AS) transfection against HDAC4, was able to revert this mechanism. In addition, since Sp3 overexpression did not change DEHP-induced HDAC4 expression, it could be suggested that in our experimental conditions Sp3 is not involved in gene regulation of the enzyme expression. Therefore DEHP-induced increase of HDAC4 mRNA levels could be due to the effect on the promoter by a transcriptional mechanism that did not involve Sp3. Furthermore, we showed that Sp3 physically interacted with HDAC4 during DEHP exposure. Interestingly,

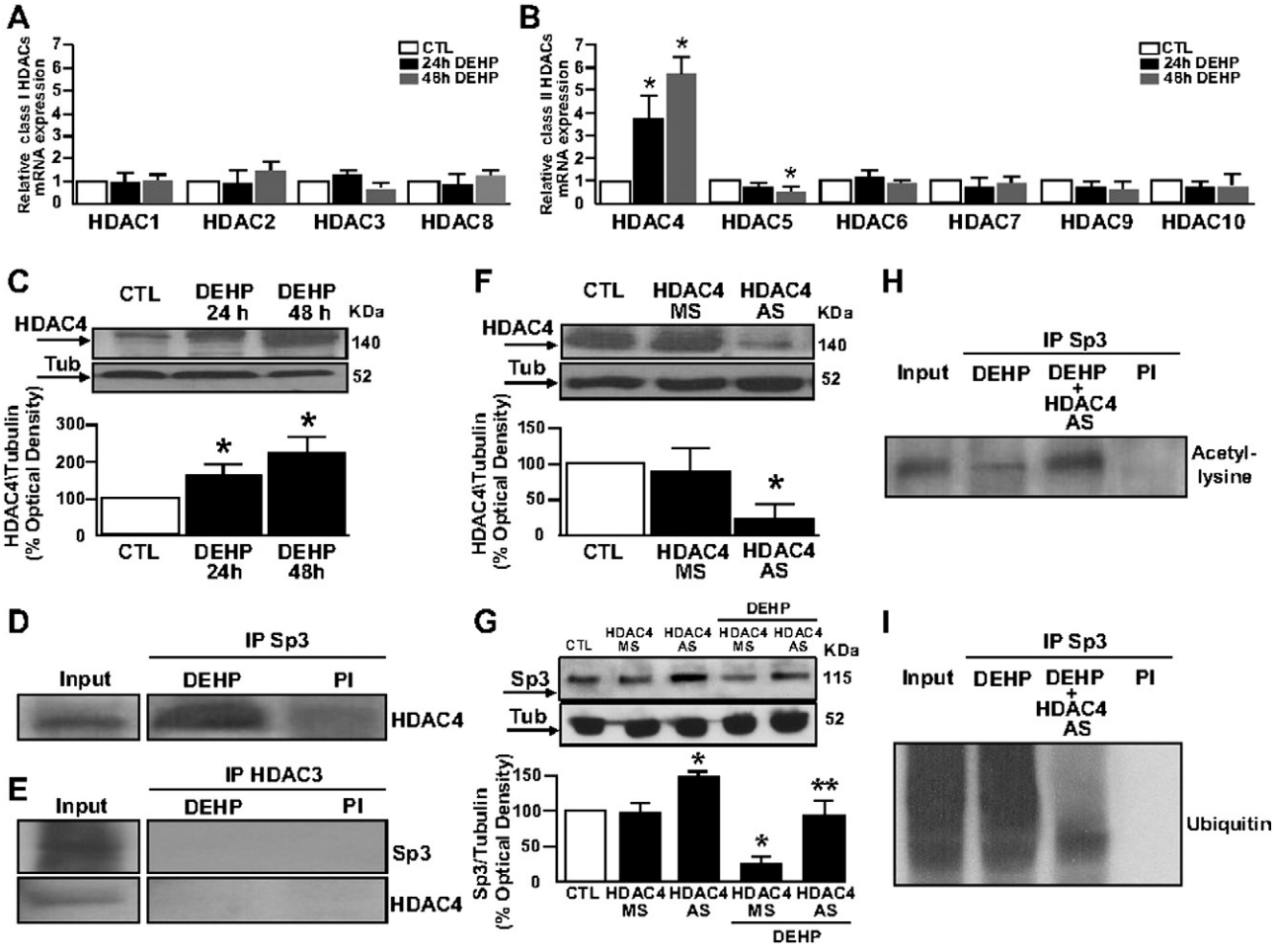

Fig. 5. Effect of HDAC4 knock-down on DEHP-induced Sp3 reduction in SH-SY5Y cells. A,B) Effect of 24 and 48 h DEHP (50 μM) exposures on HDAC/class I and II mRNA expression detected by qRT-PCR. Bars represent mean ± S.E.M. obtained from four for class I and five for class II independent experimental sessions. *P < 0.05 vs. CTL. C) Western blots of HDAC4 levels in control conditions and with 50 μM DEHP at 24 and 48 h. Graphs show the quantification of the ratio of HDAC4 to tubulin. Bars represent mean ± S.E.M. obtained from three independent experimental sessions. *P < 0.05 vs. CTL. D) Representative Western blot showing the immunoprecipitation of Sp3 and HDAC4 after 24 h treatment with 50 μM DEHP. E) Representative Western blot showing the immunoprecipitation of HDAC3 with Sp3 and with HDAC4 after 24 h treatment with 50 μM DEHP. F) Effect of antisense (AS) and missense (MS) for HDAC4 on its protein expression. G) Effect of AS and MS for HDAC4 on Sp3 protein expression, with DEHP alone (50 μM) or in combination with AS HDAC4. Bars represent mean ± S.E.M. obtained from three independent experimental sessions. *P < 0.05 vs. CTL, **P < 0.05 vs. DEHP. H,I) Immunoprecipitation with anti-Sp3 probed for HDAC4 by Western blot after 24 h treatment with DEHP alone or in combination with AS HDAC4.

DEHP-induced Sp3 binding reduction to two known Sp3 target genes Ngb (Zhang et al., 2011) and COX-2 (Lee et al., 2006) is reverted by pre-treatment with MC-1568, a well known class II HDAC inhibitor (Spallotta et al., 2013). Notably, MC-1568 also reverted DEHP-induced COX-2 and Ngb mRNA decrease, respectively. A possible mechanism by which Sp3 reduction results in DEHP-induced cell death may involve Ngb and COX-2 decrease, since both are neuroprotective (Fordel et al., 2006; Lee et al., 2006). It should be noted that in Neuro2a cells, DEHP activates Trim17 protein via PPARγ, leading to pro-caspase-3 cleavage and apoptosis, which has been postulated to impair fetal brain development (Lin et al., 2011). Collectively, the results of the present study suggest that apoptotic DEHP-induced neuronal cell death is dependent on a reduction in Sp3 protein levels which requires HDAC4. Moreover, our findings suggest that HDAC4 binding to Sp3 reduces Sp3 acetylation, while increasing its ubiquitination.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2014.07.014.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Authorship contributions

LF, NG, LMTC and GDR conceived and designed the experiments. LF, NG, GL and PM performed the experiments. LF, NG, GL, FS and PM analyzed the data. LF, NG, GL, PM, FS and MT contributed reagents/materials/analysis tools. LF, NG, LMTC and GDR wrote the paper.

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