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Inhibition of protein ubiquitination by paraquat and 1-methyl-4-phenylpyridinium impairs ubiquitin-dependent protein degradation pathways

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Abstract

Intracytoplasmic inclusions of protein aggregates in dopaminergic cells (Lewy bodies) are the pathological hallmark of Parkinson's disease (PD). Ubiquitin (Ub), alpha [α]-synuclein, p62/sequestosome 1 and oxidized proteins are major components of Lewy bodies. However, the mechanisms involved in the impairment of misfolded/oxidized protein degradation pathways in PD are still unclear. PD is linked to mitochondrial dysfunction and environmental pesticide exposure. In this work, we evaluated the effect of the pesticide paraquat (PQ) and the mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP^+) on Ub-dependent protein degradation pathways. No increase in the accumulation of Ub-bound proteins or aggregates was observed in dopaminergic cells (SK-N-SH) treated with PQ or MPP^+, or in mice chronically exposed to PQ. PQ decreased Ub protein content, but not its mRNA transcription. Protein synthesis inhibition with cycloheximide depleted Ub levels and potentiated PQ–induced cell death. Inhibition of proteasomal activity by PQ was found to be a late event in cell death progression, and had no effect on either the toxicity of MPP^+ or PQ, or the accumulation of oxidized sulfenylated, sulfonylated (DJ-1/*PARK7* and peroxiredoxins) and carbonylated proteins induced by PQ. PQ- and MPP^+-induced Ub protein depletion prompted the dimerization/inactivation of the Ub-binding protein p62 that regulates the clearance of ubiquitinated proteins by autophagic. We confirmed that PQ and MPP^+ impaired autophagy flux, and that the blockage of autophagy by the overexpression of a dominant-negative form of the autophagy protein 5 (dnAtg5) stimulated their toxicity, but there was no additional effect upon inhibition of the proteasome. PQ induced an increase in the accumulation of α-synuclein in dopaminergic cells and membrane associated foci in yeast cells.

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Our results demonstrate that inhibition of protein ubiquitination by PQ and MPP^+ is involved in the dysfunction of Ub-dependent protein degradation pathways.

Keywords

Ubiquitin-proteasome system; sequestosome 1; SQSTM1; MPP^+; ubiquitylation; autophagy; pesticides; Parkinson's disease

INTRODUCTION

Proteins are continually at risk of damage, misfolding, and aggregation. If not properly degraded, misfolded protein aggregates can cause cellular toxicity and disease. Cells have evolved an elaborated network of protein quality control mechanisms to maintain the integrity of the proteome. Protein homeostasis (proteostasis) involves specific processes that guard protein synthesis, folding and trafficking. In addition, protein degradation pathways such as the ubiquitin (Ub)-proteasome-system (UPS) and autophagy, degrade misfolded or aggregated proteins to avoid proteotoxic stress [1]. A dysfunction in protein quality control mechanisms is a hallmark in neurodegenerative diseases [2,1]. Lewy bodies (LBs) found in Parkinson's disease (PD) brains are composed of misfolded protein aggregates. A number of proteins have been identified as major components of LBs including α-synuclein, Ub and p62 [3–5]. Inhibition of proteasomal activity has been proposed to lead to the accumulation of Ub-bound proteins including α-synuclein [2]. Interestingly, other reports have demonstrated the presence of Ub-negative protein inclusions in PD brains [6–7], suggesting that different mechanisms other than impaired proteasomal activity, can be involved in the accumulation of misfolded protein aggregates.

A disruption in autophagic pathways has also been linked to PD pathogenesis [2,1,8–9]. It is now well established that protein ubiquitination (or ubiquitylation) directs the recognition of selective cargo for degradation via the autophagosome-lysosome system [10–11]. Conditional disruption of autophagy in dopaminergic cells leads to the accumulation of ubiquitinated protein aggregates *in vivo* [12–13]. Recognition of ubiquitinated proteins for their degradation by autophagy is mediated by the adapter protein p62/sequestosome 1 (SQSTM1), and the neighbor of BRCA1 gene 1 (NBR1). p62 binds ubiquitinated proteins via its Ub-associated (UBA) C-terminal domain, while its binding to autophasomal LC3/GABARAP proteins involves a short linear sequence known as LIR (LC3-interacting region) [11,14]. Interestingly, p62 also mediates the autophagic clearance of non-ubiquitinated proteins [15–16], and it may mediate the degradation of some poly-ubiquitinated proteins by the proteasome [17–18].

A large variety of oxidative protein modifications can be induced by reactive oxygen/nitrogen species, or by-products of oxidative stress. Oxidized proteins can form oligomeric complexes resulting in the formation of protein aggregates. Irreversibly oxidized proteins such as protein carbonyls have to be degraded in order to maintain proper cellular homeostasis. Ub-dependent and independent degradation of oxidized proteins by the 26S or 20S proteasome has been reported. However, covalent crosslinks, disulphide bonds, hydrophobic interactions, and heavily oxidized stable protein aggregates are not suitable for

proteasomal degradation. Recent evidence suggests that autophagy plays a major role in the removal of oxidized protein aggregates by their incomplete degradation within the lysosomal compartment that results in the formation of polymerized lipofuscin-like aggregates consisting of oxidized polypeptides [19–20]. Interestingly, p62 silencing enhances the accumulation of oxidized proteins [21], supporting a role for protein ubiquitination in the clearance of oxidized proteins by autophagy [22].

Mitochondrial dysfunction and oxidative stress are causative factors for dopaminergic cell loss in PD. Sporadic (non-hereditary) PD accounts for >80% of reported cases, while genetic mutations only account for 5% of sporadic PD occurrence [23]. Exposures to environmental toxicants, including pesticides (paraquat [PQ] and rotenone), are recognized as risk factors for an increased susceptibility to develop PD [24–29]. Thus, mitochondrial toxins such as inhibitors of complex I (1-methyl-4-phenylpyridinium [MPP+] and rotenone) and pesticides (PQ and rotenone as well) are used as toxicological models to dissect the molecular mechanisms by which mitochondrial dysfunction and oxidative stress mediate dopaminergic cell death. It has been reported that PQ and MPP^+ induce the accumulation of Ub-bound protein aggregates by impairment of the proteasomal activity [30–32]. We and others have reported that impairment of autophagy facilitates dopaminergic cell death induced by PQ and MPP^+ [33–34]. Both autophagy and the UPS are complementary protein degradation pathways where inhibition of the UPS triggers the clearance of Ub-bound proteins or aggregates by autophagy [35–36,1–2]. However, their exact and complementary contribution to dopaminergic cell death and the clearance of misfolded/oxidized protein aggregates induced by environmental/mitochondrial toxins has not been clarified.

In this work, we demonstrate that the environmental toxicant PQ and the mitochondrial complex I inhibitor MPP^+ decrease protein ubiquitination in dopaminergic cells. Inhibition of the proteasome activity was found to be a late stage during cell death progression, and did not modulate the toxicity of either PQ or MPP^+. Depletion of Ub was shown to parallel p62 dimerization/inactivation, and the accumulation of oxidized proteins and α-synuclein. Inhibition of autophagy stimulated PQ and MPP^+ toxicity. Our results demonstrate that early impairment in Ub protein synthesis by environmental and mitochondrial insults inactivates p62 and Ub-dependent degradation pathways.

MATERIALS AND METHODS

Reagents

Chloroquine, 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium iodide (MPP^+), and rotenone were obtained from SIGMA-Aldrich. 6-OHDA was prepared as described previously [37]. Paraquat (PQ, 1,1′-dimethyl-4,4′-bipyridinium dichloride) and cycloheximide (CHX) were purchased from Acros Organics. (S)-MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO) was obtained from Cayman Chemical. Pyr-41 (4-[4-[(5-Nitro-2-furanyl)methylene]-3,5-dioxo-1-pyrazolidinyl]benzoic acid ethyl ester) was purchased from Tocris. Stock solutions for MG132, Pyr-41 and rotenone were prepared in DMSO (vehicle). All other chemicals, were from SIGMA-Aldrich, Thermo Fisher Scientific or Acros Organics.

Cell culture and treatments

The dopaminergic properties of the neuroblastoma cell line SK-N-SH and their cell culture have been detailed before [33]. The Lund Human Mesencephalic (LUHMES) neuronal precursor cell line, a subclone of the tetracycline-controlled *v-myc*-overexpressing human mesencephalic-derived cell line MESC2.10, was purchased from the American Type Culture Collection (ATCC, Biosource Center). Culture of LUHMES cells was done according to Scholz *et al.*, [38]. Briefly, culture ware was pre-coated with 50 μg/ml poly-L-ornithine (SIGMA-Aldrich) and 1 μg/ml fibronectin (BD Biosciences). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 Nutrient Mixture (GIBCO or Hyclone) supplemented with Neuroplex N-2 (Gemini or Life Technologies), 2 M L-glutamine (GIBCO or Hyclone), and 40 ng/ml recombinant basic fibroblast growth factor (bFGF, Peprotech or StemRD). Cells were grown at 37°C in a 5% CO_2 humidified atmosphere. Cells were treated as indicated in the figures. Control conditions included the appropriate vehicle, which never exceed >0.01% (v/v).

Protein extraction and western immunoblot (WB)

Protein extraction and quantification were done as explained before [39]. For the detection of sulfenic acid modified proteins (PSOH) cells were incubated with the cell-permeable nucleophilic reagent dimedone prior to harvesting. Dimedone selectively reacts with the electrophilic sulfur atom in sulfenic acid to form a stable thioether that can be detected using the anti-sulfenic acid modified 2-thiodimedone specific antibody [40]. The protein carbonyl content was determined by the reaction of carbonyl groups in protein side chains with 2,4–dinitrophenylhydrazine (DNPH, SIGMA-Aldrich) to form 2,4-dinitrophenylhydrazone (DNP), which is detected using anti-DNP antibodies (SIGMA-Aldrich). Cells were lysed in the presence of 1 mM DTPA (SIGMA-Aldrich). Ten μg of protein were dissolved with a final concentration of 6% sodium dodecyl sulfate (SDS) w/v, and were derivatized by the addition of 20 mM DNPH in 10% trifluoroacetic acid (TFA, w/v) [41–42]. After derivatization, samples were neutralized with 2 M Tris, 30% Glycerol (v/v) and 10.2% β-mercaptoethanol (v/v) to obtain a final concentration of 2.8% (v/v).

Polyacrylamide gel electrophoresis (PAGE) was performed using Bis-Tris (with 3-(N-morpholino) propansulfonic acid [MOPS] + 5 mM sodium bisulfite-based running buffer), or Tris-Glycine gels. Proteins were transferred to nitrocellulose (GE Healthcare Life Sciences) or PVDF membranes (Maine Manufacturing). Blots were blocked and incubated with the corresponding primary antibodies as recommended by the manufacturers: anti-Ub P4D1 (Cat # 3936) and α-synuclein (Cat # 2642, carboxy-terminal sequence) were from Cell Signaling; anti-green fluorescent protein (GFP, Cat # 1020) was from Aves Labs; anti-SQSTM1/p62 (Cat # Ab109012), anti-sulfonylated peroxiredoxins (Prx-SO_3H, Cat # ab16830), and anti-sulfonylated DJ-1 (DJ-1-SO_3H, Cat #ab169520) were from Abcam; anti-PSOH modified 2-thiodimedone-specific antibody (Cat #ABS30) was from Millipore; and anti-DNP (Cat # D9656), and anti-microtubule-associated protein 1B-light chain (3LC3B, Cat # L7543) were from SIGMA-Aldrich. Blots were probed with β-actin (Cat #A2228, SIGMA-Aldrich) to verify equal protein loading. Peroxidase conjugated secondary anti-rabbit, anti-mouse or anti-chicken antibodies (Thermo Scientific or Cell Signaling Technology) were used and bands were detected using enhanced chemiluminescence (ECL)

western blotting substrate (Thermo Scientific/Pierce or Amersham/GE Healthcare Life Sciences) in a C DiGit Chemiluminescence Western Blot Scanner (LI-COR Biosciences) or a VersaDoc Gel Imaging System (Bio-Rad).

Analysis of high molecular weight aggregates (HMW) of α-synuclein from soluble and insoluble fractions (SDS-PAGE), and native α-synuclein conformation by Blue Native-polyacrylamide gel electrophoresis (BN-PAGE) from Triton (X-100) insoluble fractions was performed as explained before [43].

Filter trap assay for ubiquitinated protein aggregates

Cells were harvested as explained before, and proteins were denatured in lithium dodecyl sulfate (LDS)-sample buffer. Ten to 50 μg of protein were filtered in a nitrocellulose membrane previously equilibrated in transfer buffer using a dot blotter (Scie-Plas). Membranes were washed twice with 2% SDS (w/v), 10 mM Tris-EDTA, pH 7.5 buffer and Ub-bound protein aggregates were detected by immunoblotting [44].

Cell death determination (loss of plasma membrane integrity) and oxidative stress

Loss of cell viability was determined using flow cytometry by measuring propidium iodide uptake (PI, 1 μg/ml) (Life Technologies or SIGMA-Aldrich) as a marker of plasma membrane integrity loss. Flow cytometry was performed as explained before [45–46,37].

Evaluation of ubiquitin-dependent and -independent protein degradation

The plasmid encoding the Ub-dependent (GFPμ) fluorescence reporter was kindly provided by Dr. Ron Kopito (Stanford University) [47]. GFPμ consists of the fusion of a 16 amino acid CL1 degron (a degradation signal identified in yeast) with the carboxyl terminus of GFP. CL1 targets GFP for ubiquitination, aggregation and degradation by the proteasome [47]. The GFPμ plasmid was linearized with *Nde I* and transfected into SK-N-SH cells using FuGENE HD reagent (Promega). Cells stably overexpressing GFPμ were selected in medium containing 0.3 mg/ml geneticin (G418, Acros Organics), and GFPμ positive cells were sorted in a FACSAria cell sorter (BD Biosciences). After treatment, cells were harvested in phosphate-buffered saline (PBS) and analyzed by flow cytometry. GFPμ was excited with a 488 nm laser and the emission was detected through a 530/30 emission filter in a FACSort (BD Biosciences / Cytek-DxP-10 upgrade) flow cytometer. The geometric mean of GFPμ fluorescence intensity was assessed in viable cells (PI negative [-]).

Determination of proteasomal activity in total cell lysates

Total cell lysates were prepared on ice by homogenization in radioimmunoprecipitation assay buffer (RIPA: 50 mM Tris-HCl pH 8.0, 150 mM $NaCl_2$, 1.0% Igepal [NP-40, v/v], 0.5% sodium deoxycholate [w/v], 0.1% SDS [w/v]). Lysates were cleared by centrifugation and protein content was determined by the bicinchoninic acid assay (BCA) method. The chymotrypsin-like activity was measured using the Proteasome Activity Assay Kit (Abcam 107921) that utilizes a 7-Amino-4-Methylcoumarin (AMC)-tagged peptide substrate (Succ-LLVY-AMC), which upon cleavage, releases free fluorescent AMC. Proteasomal-mediated AMC release was measured kinetically for 120 min using a microplate reader (TECAN, excitation/emission of 350/440 nm) in the presence or absence of MG132. Results were

expressed as U/ml/mg of protein, where one unit (U) of proteasome activity is defined as the amount of proteasome that generates 1.0 nmol of AMC per minute (nmol/min).

Evaluation of cellular proteasomal activity

After treatment, cells were incubated with the boron-dipyrromethene (Bodipy)-tagged cell-permeable proteasome activity probe BodipyFL-Ahx3L3VS (200 nM) for 2 h, which was synthesized as explained before [48–49]. Cells were harvested in PBS. BodipyFL-Ahx3L3VS was excited with a 488 nm laser and the emission was detected through a 530/30 emission filter in a BDFACSort (Cytek-DxP-10 upgrade). The geometric mean of BodipyFL-Ahx3L3VS fluorescence intensity was assessed to evaluate changes in fluorescence that directly relate to proteasome activity.

Evaluation of ubiquitin B (*UBB*) mRNA levels

RNA was extracted with Trizol (Life Technologies) following the manufacturer's instructions and quantified in a Nanodrop 2000 (Thermo Scientific). cDNA strands were synthesized using 5 μg of RNA and Moloney Murine Leukemia Virus Reverse Transcriptase M-MLV (200 U/μL, Life Technologies), Oligo (dT) primer (0.5 μg/μL, Life Technologies) and dNTPs (2.5 mM each, Applied Biosystems) during one cycle of amplification under the following conditions: 1) 65 °C / 5 min, 2) 37 °C / 50 min, and 3) 70 °C / 15 min (Applied Biosystems 2720 Thermal Cycler). The *UBB* mRNA expression levels were determined by real time PCR (RT-PCR) using 100 ng of cDNA as template, TaqMan Universal PCR Master Mix (Applied Biosystems), and specific TaqMan probes for the human poly-ubiquitin gene *UBB* (Hs00430290_m1 FAM, Applied Biosystems). The probe used to detect *UBB* mRNA amplifies 6 different variants (NM_018955 and NM_001281716-9). RT-PCR was performed in an ABI Prism 7500 (Applied Biosystems) under the following conditions: step 1) 50°C / 2 min; step 2) 95°C / 10 min; and step 3) 40 cycles of 95°C / 15 sec, followed by 60°C / 1 min. Data were normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991_g1 VIC) as endogenous housekeeping gene by the relative standard curve method (http://www.uic.edu/depts/rrc/cgf/realtime/stdcurve), and the results were expressed as relative expression levels with respect to the control group.

Recombinant adenoviral vectors

The replication-deficient recombinant adenovirus (Ad5CMV) encoding a dominant negative form (dn) of the autophagy protein 5 (ATG5) was kindly provided by Dr. Gökhan S. Hotamisligil (Harvard School of Public Health, Boston, MA) [50]. Adenoviruses encoding wild type (WT) or mutant A53T α-synuclein were provided by Dr. Jean-Christophe Rochet (Purdue University) and have been described elsewhere [51,43]. Adenovirus containing only the CMV promoter (Ad-Empty) was used as a negative control. Viruses were amplified and tittered in HEK293T cells as previously described [52,39]. Cells were infected with viral particles at the indicated multiplicity of infection (MOI), and 24 h post-infection, they were washed and treated under the specified experimental conditions.

In vivo mouse model of paraquat toxicity

C57BL/6 mice (8–10 weeks old) (Jackson Labs) were administered two intraperitoneal injections of 10 mg/kg PQ or PBS every week for 9 consecutive weeks [53]. Animals were analyzed 1 week after the last injection. Mice were decapitated and midbrains were removed for WB analysis. For immunohistochemistry, mice were perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed, post-fixed for 24 h in 4% PFA and cryoprotected with 30% sucrose. Frozen brains were cut into 30 μm coronal sections using a sliding microtome at −16°C, and stored in PBS at 4°C until the immunohistochemical procedure. Endogenous peroxidase activity was inactivated. Sections were blocked with 10% normal horse serum (Life Technologies) and incubated 48 h with anti-tyrosine hydroxylase antibody (TH, Calbiochem, EMD/Millipore Cat # AB1542) or anti-Ub at 4°C. After rinsing, sections were incubated in secondary Alexa 647-anti-mouse or Alexa 568-anti-sheep (Jackson ImmunoResearch) for 1 h at RT. Sections were mounted with Fluoro-gel (Electron Microscopy Sciences) containing 4’,6-diamidino-2-phenylindole (DAPI) to label nuclei. Images were collected on an Olympus IX 81 inverted confocal scanning fluorescent microscope (10x or 60x oil lens) (Olympus America) using Fluoview 500 Software. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln (Project 1025)

Yeast experiments

Saccharomyces cerevisiae W303-1A strain (*MATa can1-100 ade2-1his3-1,15 leu2-3,112 trp1-1 ura3-1*) harboring chromosomally integrated human α-synuclein-GFP expression cassette under the control of the inducible *GAL1* promoter was generated and handled as previously described [43]. For confocal microscopy, live cells were visualized with 100x oil lens. For survival assays, aliquots of the yeast culture were diluted to 300 cells and plated onto YP (yeast peptone) plates containing 2% glucose (YPD, w/v) or 2% galactose (YPGal, w/v) as the sole carbon source. The colony forming units or degenerative colonies were scored following 2 (YPD plates) or 4 (YPGal plates) days of incubation at 28 °C.

Statistical analysis

Experimental replicas were independent and performed on separate days. Collected data were analyzed by using one-way, two-way or three-way ANOVA, and the appropriate post-hoc test using SIGMA-PLOT/STAT package. When ANOVA assumptions were not met (normality [Shapiro–Wilk test] or equal variance) Kruskal-Wallis one-way ANOVA on Ranks or data transformation (two-way ANOVA) were performed on the collected data. Data were plotted as mean ± standard error (SE) using the same package for statistical analysis. Flow cytometry plots and immunoblots presented show the results of representative experiments. Relative densitometry analysis of WBs and dot blots was made using the ImageJ Program (National Institutes of Health, http://rsb.info.nih.gov/ij).

RESULTS

Effect of PD-related toxicants on the accumulation of Ub-bound proteins

PD is linked to mitochondrial dysfunction and environmental pesticides exposure [54–55]. Previous reports have demonstrated that PD-related toxicants impair the activity of the proteasome leading to the accumulation of Ub-bound protein aggregates [30–32,56–57]. We found that exposure of dopaminergic neuroblastoma cells (SK-N-SH) to PQ induces a dose-dependent decrease in ubiquitinated protein levels (Suppl. Fig. 1a). Lower non-toxic concentrations of PQ (≤0.2 mM) induced no changes in the levels of ubiquitin-bound proteins (Suppl. Fig. 1a). Higher toxic concentrations of PQ (≥0.5-1 mM) induced a slight decrease in ubiquitinated proteins (Suppl. Fig. 1a). Similarly, PQ induced a dose dependent decrease in the accumulation of Ub-bound proteins in the mesencephalic neuronal precursor cell line LUHMES (Suppl. Fig. 1a), whose sensitivity to PQ is significantly higher (~100 to 200 μM, *not shown*). These results demonstrate that the decrease in ubiquitinated protein levels induced by PQ is not cell type specific.

A decrease in ubiquitinated proteins can be ascribed to different phenomena including an enhanced proteasomal activity, a decrease in protein ubiquitination, and/or a reduced availability of free Ub monomers/chains. To test this possibility, cells were treated with PQ and incubated with the cell permeable proteasome inhibitor MG132 (0.2 μM) 24 h prior to analysis (see Fig. 1a *upper box*). As previously reported [43], at this concentration and time of incubation MG132 efficiently inhibits the proteasome activity leading to the accumulation of Ub-bound proteins without triggering significant cell death by itself (Suppl. Fig. 1b and Fig. 2a–b). PQ induced a dose-dependent decrease in Ub-bound proteins even in the presence of MG132 (Fig. 1a, *quantified in* 1b [with respect to control] *and in* Suppl. Fig. 1c [with respect to each treatment in the absence of MG132]), suggesting that the decreased accumulation of ubiquitinated proteins is ascribed to a reduction in protein ubiquitination. Similarly, treatment with the mitochondrial toxin MPP^+, but not rotenone or 6-OHDA, also reduced protein ubiquitination (Fig. 1c).

A dysfunction in the UPS has been shown to lead to the formation of SDS-resistant aggregates (aggresomes) [58–59]. Protein complexes and aggregates are not well resolved by WB due to their high molecular weight. Thus, we evaluated the changes in both ubiquitinated proteins and aggregates by filter trap (retardation) dot blot assay. Fig. 1d corroborates that both PQ and MPP^+ induce a decrease in protein ubiquitination, evaluated in the presence of MG132. Finally, to evaluate the effect of PQ exposure on the levels of Ub-bound proteins in dopaminergic cells *in vivo*, C57Bl/6 mice were exposed chronically (9 weeks) to PQ. Immunohistochemistry analysis of TH+ neurons and Ub shows that no major increase in Ub staining was induced by PQ in dopaminergic (TH+) cells (Fig. 1e).

We have previously demonstrated that cell death induced by PQ is a stochastic process [33,45,39], which means that it occurs at different rates within the same cell population. As such, WB analysis does not allow us to determine if the changes in Ub-bound protein levels occur before or after cell death (i.e. samples are composed by a mixed population of cells at different stages during the cell death process). Thus, we next evaluated the accumulation of GFPμ, a substrate for the UPS, in live cells (*as depicted in broken square regions in* Fig. 2a

and c). Treatment with MG132 induces the accumulation of GFPμ (Fig. 2a–b). Lower non-toxic concentrations of PQ (≤ 0.2 μM) induced a significant accumulation of GFPμ (Fig. 2c–d), which agrees with the overall decrease in protein ubiquitination previously seen (Fig. 1a–b, *PQ + MG132 data*). Surprisingly, while higher toxic concentrations of PQ decrease protein ubiquitination further (Fig. 1a–b) this was not translated in added accumulation of GFPμ (Fig. 2c–d). A time-course analysis of changes in GFPμ levels induced by a toxic concentration of PQ (0.5 mM, 48 h, Suppl. Fig. 2a), or a sub-toxic PQ concentration (0.1 mM, 96 h, Fig. 2e) also evidenced an early accumulation of GFPμ followed by a reduction in its levels in live cells irrespective to the dose of PQ used or the length of exposure. Similarly, MPP^+, which also impairs protein ubiquitination (Fig. 1c), also induced a reduction in GFPμ content (Suppl. Fig. 2b–c). Poly-ubiquitin tagged proteins have a half-life ($t_{1/2}$) of ≤ 30 min [60]. The GFPμ reporter has a reported short-term $t_{1/2}$ = 30 min [61–62], while GFP itself has a $t_{1/2}$ = 24 h [63]. Thus, our results suggest that while the transient accumulation of UPS substrates (GFPμ) induced by PQ is linked to an impairment of protein-ubiquitination (Fig. 2d–e), the subsequent Ub-independent decrease in GFPμ induced by both PQ and MPP^+ is likely linked to impaired protein synthesis.

Impairment of proteasomal activity by PQ is a late step in the cell death process

Previous studies have demonstrated that PD-related toxicants including PQ and MPP^+ impair proteasomal activity [64–65,30–31,57]. We found that the activity of the proteasome decreased in lysates from cells treated with toxic PQ concentrations (≥ 0.5 mM) (Fig. 3a). However, due to the stochastic nature of cell death progression, this assay does not allow us to evaluate if changes in the activity of the proteasome occur prior to cell death, or if they are only an epiphenomenon associated with the loss of cellular viability. Thus, we evaluated the changes in the activity of the proteasome in intact cells using the cell permeable proteasome activity probe BodipyFL-Ahx3L3VS [49,48]. MG132 inhibits the processing of BodipyFL-Ahx3L3VS by the proteasome (Fig. 3c and Suppl. Fig. 3a). PQ (Fig.3b and 3c) and MPP^+ (Suppl. Fig. 3b) induced a dose-dependent increase in proteasome activity. Accordingly, previous studies have reported that PQ and MPP^+ induce an early increase in proteasomal activity [66–68]. Thus, our results suggest that the impairment in proteasomal activity induced by PQ and other PD-related insults is a late event associated with the loss of cell viability. Accordingly, MG132 had no effect on PQ-, MPP^+-, rotenone- or 6-OHDA-induced toxicity (Fig. 3d).

PQ reduces Ub-protein content but not Ub-mRNA transcription

The decrease in protein ubiquitination induced by PQ and MPP^+ might be mediated by impairment in the activity of Ub-activating (E1s) and Ub-conjugating enzymes (E2s), and/or Ub-ligases (E3s). A decrease in the activity of these enzymes is translated in the accumulation of free Ub monomers/chains [69]. However, we found that treatment of cells with PQ induced a decrease in Ub-monomers (Fig. 4a and Suppl. Fig. 3c), suggesting that a decrease in the Ub-protein pool, rather than an impairment in the E1-E2-E3 system, is linked to the reduced levels of protein ubiquitination. Ub is encoded in mammals by 4 genes. *UBA52* and *RPS27A* genes code for a single copy of Ub fused to ribosomal proteins, while the *UBB* and *UBC* genes code for poly-Ub precursor proteins. We evaluated if the reduction in Ub protein levels induced by PQ is mediated by a decrease in gene transcription.

Surprisingly, a significant increase in *UBB* mRNA transcription/stability was observed at lower concentrations of PQ (≤ 0.2 mM) (Fig. 4b). These results demonstrate that Ub-protein synthesis or stability, but not Ub-gene transcription, is impaired by PQ.

We next evaluated the role of Ub-protein synthesis inhibition/depletion in PQ toxicity. Because Ub is encoded by 4 genes, its knockdown is experimentally cumbersome as reported in previous studies demonstrating that while *UBB* knockdown reduces Ub monomers by 70%, it only decreases Ub-bound proteins by 30% [70]. A previous study demonstrated that the protein synthesis inhibitor cycloheximide (CHX) depletes cellular Ub resulting in a decrease in steady-state poly-ubiquitinated proteins [71]. Depletion of Ub-bound proteins was induced by CHX treatment (48 h, Fig. 4c), which only resulted in a slight increase in cell death (Fig. 4d). CHX significantly stimulated PQ toxicity (Fig. 4d), suggesting that Ub depletion, but not inactivation of the proteasome, contributes to PQ toxicity. Ub depletion by itself does not induce cell death, suggesting that additional events linked to PQ or MPP^+ exposure (oxidative damage or mitochondrial dysfunction) in addition to impaired protein ubiquitination are required for cell death progression.

PQ-induced oxidized protein accumulation is not regulated by the proteasome

Clearance of oxidized proteins has been shown to be mediated by both Ub-dependent and -independent proteasomal degradation pathways [72–73]. PQ induced a dose-dependent accumulation of sulfenylated protein cysteine residues (PSOH), irreversibly oxidized (sulfonylated PSO_3H) DJ-1 and peroxiredoxins (Prxs), and protein carbonyls (Fig. 5a–c and Suppl. Fig. 4a–b). However, inhibition of the proteasome with MG132 did not increase further the accumulation of oxidized protein byproducts (Fig. 5a–c and Suppl. Fig. 4a–b), suggesting that the increased load in oxidized proteins is ascribed to impaired protein ubiquitination but not to a decrease in proteasomal activity. We have previously demonstrated that oxidative stress induced by MPP^+ is primarily restricted to the mitochondria matrix [45]. Accordingly, no accumulation of oxidized DJ-1 or Prxs was observed upon exposure to MPP^+.

Dimerization of p62 parallels the impairment in protein ubiquitination and autophagy flux

Selective degradation of ubiquitinated protein aggregates is also mediated by autophagy via the Ub binding receptor p62 [10]. p62 binds to ubiquitinated proteins via its UBA C-terminal domain. Interestingly, the UBA domain, which has a low affinity for Ub, also mediates the formation of highly stable symmetrical inactive dimmers. p62 dimerization and Ub-binding are mutually exclusive [74]. Thus, we considered that Ub depletion induced by PQ might dimerize/inactivate p62. Inhibition of Ub activating enzymes (E1s) with Pyr 41 induced p62 dimerization (Fig. 6a and Suppl. Fig. 5a) [75]. PQ, and to a lesser extent MPP+ and 6-OHDA, but not rotenone also induced a dose-dependent dimerization of p62 (Fig. 6b and Suppl. Fig. 5b–c). Overexpression of a dominant negative form of ATG5 (dnATG5), which together with ATG12 and ATG16 is essential for autophagosome formation [76], enhanced p62 dimerization (Fig. 6b and Suppl. Fig. 5b). Overexpression of dnATG5 did not impair the decrease in ubiquitinated proteins/aggregates induced by PQ demonstrating that Ub-protein depletion parallels p62 dimerization (Fig. 6c).

The accumulation of autophagosomes is evidenced by an increase in the levels of the microtubule–associated protein light chain (LC3–I) protein in its lipidated form (LC3–II). As previously reported [77,36,78], inhibition of the proteasome with MG132 induces autophagy (Suppl. Fig. 5d). We and others have previously demonstrated that PQ and the complex I inhibitors MPP^+ and rotenone impair autophagy flux [33–34,79], defined as the complete process beginning with the formation of the phagophore, and ending after the fusion of the autophagosomes with the lysosome for the degradation of lysosomal cargo [46,9]. Autophagy flux was inferred by WB analysis of LC3–II turnover in the presence of cloroquine (CQ), the inhibitor of lysosomal cargo degradation that specifically inhibits the acid-dependent breakdown of autolysosome content without affecting autophagosome–lysosome fusion, which results in the accumulation of autophagolysosomes that cannot be cleared [46]. Fig. 6d and Suppl. Fig. 5e–f corroborate that PQ induces a dose- and time-dependent impairment in autophagy flux. We have previously demonstrated that overexpression of dnATG5 inhibits autophagy and potentiates PQ and MPP^+ toxicity [33]. Protein degradation mechanisms are complementary, and dysregulation of either the UPS or autophagy has been reported to be mutually compensated, particularly in the clearance of aggregated proteins linked to neurodegenerative disorders [35–36,80–85]. While inhibition of autophagy with dnATG5 overexpression stimulated PQ toxicity, MG132 exerted no additional toxicity when combined with dnATG5 overexpression Fig. 6e. These results demonstrate that Ub-protein depletion induced by PQ and MPP^+ is linked to the inactivation of p62 that precedes the decrease in autophagy flux. Autophagy but not the proteasome regulates the progression of PQ and MPP^+-induced dopaminergic cell death.

Paraquat increases the pathological accumulation of α-synuclein in dopaminergic cells and membrane-associated foci in yeast

Clearance of misfolded/aggregated α-synuclein has been shown to be mediated by both autophagy and the ubiquitin/proteasome pathways [86,84–85]. Previous studies have reported that PQ upregulates the levels of α-synuclein [87–90,34]. We observed no effect of PQ on the total endogenous levels of α-synuclein in SK-N-SH dopaminergic cells (*data not shown*) or in PQ treated C57Bl/6 (Fig. 7a). Thus we evaluated if PQ could alter the pathological accumulation of α-synuclein when overexpressed (as a PD model of *SNCA* multiplication) or when mutated. Overexpression of α-synuclein (WT or A53T mutant) in SK-N-SH cells for 72 h, in the presence or absence of PQ did not induce the accumulation of HMW aggregates of α-synuclein (SDS-PAGE analysis of whole cell lysates containing soluble and insoluble fractions), but it increased the accumulation α-synuclein in its monomeric form (Fig. 7b). α-synuclein is reported to exist predominantly as stable unfolded monomers that migrate as 57–60 kDa proteins (Fig.7c). It is unclear whether the larger than expected size of the bands is a result from the monomers adopting an unfolded extended conformation, which results in a larger than expected hydrodynamic radius [91–93], or if it represents a fraction of α-synuclein existing as a stable tetramer [94–95]. We have previously demonstrated that inhibition of the proteasome promotes the accumulation of α-synuclein in this unfolded state (or tetramer) and the appearance of a band with enhanced lower MW [43]. Similarly, PQ induced the accumulation of unlfoded α-synuclein (Fig. 7c) and the accumulation of a low MW band immunoreactive for α-synuclein (depicted with * in Fig. 7c).

To further interrogate the effect of PQ on α-synuclein distribution we used the budding yeast *S. cerevisiae* genetic model overexpressing an inducible promoter-driven fusion of α-synuclein-GFP. Yeast has been extensively used as a valid experimental platform to elucidate the fundamental mechanisms associated with neurodegenerative diseases [96]. The inducible expression of α-synuclein can result in no toxicity, intermediate toxicity, or high toxicity in relation to the levels of α-synuclein being expressed. α-synuclein-GFP overexpressed in yeast at non-toxic levels localizes at the plasma membrane, consistent with its known affinity to phospholipids (Fig. 7d). This is the expected localization of a protein that localizes at synaptic vesicles in neurons, when considering that yeast has constitutive vesicular secretion [96]. Acute or chronic treatment of yeast cells with PQ induced the accumulation of membrane associated foci (Fig. 7d). While α-synuclein overexpression had no effect in SK-N-SH (*not shown*) or yeast cell death induced by PQ (Fig. 7e), in yeast it induced the formation of degenerative colonies (Fig. 7e) (smaller in size and impeded in their ability to propagate normally). These results suggest that the accumulation of monomeric α-synuclein and its localization at the plasma membrane is regulated by PQ.

DISCUSSION

The etiology of PD involves the convergence of aging, genetic and environmental risk factors [54,97]. A disruption in protein quality control mechanisms is linked to the accumulation of protein inclusions and neuronal cell loss observed in neurodegenerative disorders including PD [98,2]. Ub selectively targets cargo to the three major protein degradation pathways, the proteasome, the lysosome, and the autophagosome [99], and environmental/mitochondrial toxicants inhibit the activity of the proteasome and impair autophagy flux [57,33]. However, the effects of environmental/mitochondrial toxicants on other components of Ub-dependent protein degradation pathways have not been studied in detail, and inhibition of the proteasome is still considered the major mechanism involved in the impairment of the Ub-dependent degradation of misfolded/damage proteins. In this work, we demonstrated that impaired protein ubiquitination is an early and central step in the impairment of protein degradation pathways induced by PQ and MPP^+. The depletion of the Ub protein pool induced by both agents was observed at both sub-toxic and toxic (subchronic) exposures and was not cell type specific. Furthermore, Ub-protein depletion was paralleled by the inactivation of p62 and in the case of PQ, the accumulation of oxidized protein byproducts and alterations in the levels of monomeric α-synuclein and its distribution at the plasma membrane (Fig. 8). These results might explain the heterogeneity of protein inclusions in PD brains (LBs), particularly, the presence of Ub–negative protein inclusions [6–7].

A decrease in the activity of the proteasome has been found in PD brains [100–103]. Previous studies have also demonstrated that environmental and mitochondrial toxins including PQ and MPP^+ impair the activity of the proteasome by: 1) direct inhibition of the proteasome, 2) mitochondrial dysfunction and energy depletion, or 3) oxidative stress [64,30,104,31,57]. Similar to previous reports [34,30,105,31] we observed that high and toxic PQ concentrations induced a decrease in the chymotrypsin-like activity of the proteasome. However, biochemical assays of proteasomal activity are usually done in lysates from cell samples (or PD tissues), where the decrease in proteasome activity might be

confounded by the loss of cells (viability). Using a novel cell-permeable proteasomal substrate (BodipyFL-Ahx3L3VS), we found that both sub-toxic and toxic concentrations of PQ and MPP^+ increase the activity of the proteasome prior to cell death. Interestingly, previous studies have demonstrated that MPP^+-induced dopaminergic cell death actually requires an increase in the activity of the proteasome [66–67]. Our findings do not argue against previous studies demonstrating that impairment in proteasome activity is induced by PD-related toxicants [64,30,104,31,57]. However, our results suggest that inhibition of the proteasome by environmental / mitochondrial toxicants might be a late event only ascribed to the loss of cell viability. As such, inhibition of the proteasome had no effect on PQ or MPP^+ induced toxicity. Accordingly, previous *in vivo* studies using the MPP^+ precursor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) found no stimulating effect of proteasome inhibition on dopaminergic cell death [106].

Previous reports have demonstrated that PD-related toxicants induce the accumulation of Ub-bound protein aggregates, which has been ascribed primarily to proteasome inhibition by these agents [30–32,104,56–57]. *In vivo*, MPTP has been shown to induce both an increase and a decrease in the levels of Ub-bound proteins using different experimental paradigms [32,107]. While we did observe a slight increase in the accumulation of the UPS fluorescent substrate GFPμ, this transient increase seems to precede the late decrease in proteasomal activity. Proteasomal activity is very robust and GFPμ accumulation induced by proteasome inhibitors has been reported to require about 70% inhibition of proteasomal activity [47]. Thus, our results are more consistent with the notion that the transient increase in GFPμ accumulation is primarily linked to impaired ubiquitination and not to the inhibition of the proteasome.

We demonstrated here that the primary effect of acute or prolonged treatment with PQ and MPP^+ at either high (toxic) or low (sub-toxic) doses is a decrease in protein ubiquitination (Fig. 8). We and others have previously shown that chronic inhibition of the proteasome depletes cells from Ub [108,43,109]. Bence *et al.* found that the GFPμ fluorescence rapidly declined in cells after the exposure to a protein-synthesis inhibitor due to the impaired synthesis of short–lived proteins such as GFPμ [61–62]. However, PQ-induced Ub-protein depletion seems to precede the inhibition of the proteasome, discarding a possible negative feedback loop from the proteasome to Ub-protein synthesis. Interestingly, while MPP^+ reduced protein ubiquitination, rotenone (another complex I inhibitor) had no such effect, which adds to the cumulative evidence that the toxicity induced by MPP^+ and rotenone might actually involve different mechanisms [33,45,110–115].

Levels of endogenous Ub-conjugates depend on the balance between 1) the rate of Ub conjugation determined by the availability of Ub, the activity of the E1-E2-E3 system and ATP levels; and 2) the rate of the turnover of Ub-conjugates (degradation/deubiquitination), which depends on the activity of the proteasome, autophagy and deubiquitinating enzymes [99]. Several components of the UPS can present different sensitivities to oxidative damage/modulation [116–123]. Deubiquitinating enzymes are inhibited by oxidation of their catalytic cysteine residue [124–125]. The proteasome has been reported to be more susceptible to oxidative inhibition than Ub-conjugating enzymes [126,116,127]. Accordingly, mild to moderate oxidative stress upregulates Ub and the Ub-conjugating

system promoting the formation of Ub-conjugates, and reducing the activity of the proteasome. In contrast, extensive but not lethal oxidative stress reduces the formation of Ub-conjugates by inactivating Ub–conjugating enzymes promoting the accumulation/aggregation of damaged/abnormal proteins [126]. While it is possible that PQ might interfere with the activity of the Ub-conjugating system (E1, E2 and E3s), this should have been translated into the accumulation of Ub monomers/free chains. In contrast, PQ clearly depleted cells from Ub even at sub-toxic concentrations. Thus, our results imply that PQ-induced depletion of Ub-bound proteins is associated with a decrease in the Ub-protein pool.

While PQ clearly reduced the Ub-protein pool, Ub mRNA levels were shown to increase in response to PQ, which is consistent with the notion that Ub is a stress-inducible protein [70,128]. These findings suggest that PQ or MPP^+ might impair the synthesis of Ub at the post-transcriptional level or modify Ub protein stability. Protein synthesis has been shown to be more sensitive to oxidative stress than DNA/RNA synthesis [129]. Ub-protein depletion induced by PQ and MPP^+ was also paralleled by a decrease in GFPμ fluorescence in stable cells. Thus, our results indicate that PQ and MPP^+ are likely impairing overall protein synthesis, which should initially affect short-lived proteins such as Ub and GFPμ (Fig. 8). MPP^+ has been previously shown to inhibit protein synthesis [130], but the mechanisms involved remain unknown. PQ and MPP^+ induce oxidative stress and energy failure, which can affect overall protein synthesis. The correct attachment of amino acids to each tRNA species that is required for protein synthesis is an energy-dependent process carried out by aminoacyl-tRNA synthetases. Additionally, aminoacyl-tRNA synthetases also hydrolyze (edit) an incorrectly attached amino acid, and oxidative stress induces protein mistranslation and degradation by impairment of aminoacyl-tRNA synthetase editing [131]. Oxidative stress also diverts tRNA synthetases to the nucleus to protect against oxidative damage [132]. Moreover, oxidized mRNA, protein mistranslation and subsequent degradation have been recently recognized as important contributors to neurodegeneration [133–138]. To determine whether energy failure, oxidative stress or both impair Ub-protein synthesis will require further investigation. However, we have demonstrated here and in previous studies that oxidative stress induced by MPP^+ and low PQ concentrations is primarily ascribed to mitochondria [45], suggesting that energy failure might be the primary mechanism involved in impaired protein (Ub) synthesis.

An increased accumulation of oxidized protein byproducts is found in PD brains. Elevated levels of carbonylated proteins [139] and cysteine oxidized proteins including the hydrogen peroxide scavengers Prxs and the early onset PD-related protein-gene DJ-1/*PARK7* [140–142] are important oxidative biomarkers detected in PD brains. We observed that PQ induced an increase in the accumulation of PSOH, precursors of irreversibly oxidative protein sulfinic (PSO_2H) and PSO_3H acid modifications. Accordingly, PQ also induced a dose-dependent accumulation of irreversibly oxidized DJ-1-SO_3H and Prxs-SO_3H, as well as protein carbonyls. Both DJ-1 and Prx turnover has been proposed to be mediated by the UPS [143–145]. Cysteine sulfenylation at the N-terminus of proteins is an important step in the endoproteolytic cleavage and formation of N-degrons recognized by the UPS [146]. Both Ub-dependent and Ub-independent degradation of oxidized proteins has been reported [72–73,126,147,22]. Accumulation of oxidized proteins in the absence of Ub-bound protein aggregates suggests that ubiquitination might be required for the degradation of oxidized

proteins induced by PQ. Aging is the main risk factor in the development of PD [148]. Similar to our results, it was observed that in an aging yeast model poly-ubiquitinated proteins are significantly reduced even in the presence of a decrease in proteasomal activity leading to an increase/accumulation of oxidized proteins [149]. Moreover, a decrease in Ub-conjugates as well as in *de novo* Ub conjugation activity was reported in lenses from aged rats, and these effects were associated with the accumulation of damaged proteins [150].

It has been recently recognized the important role that autophagy plays in the degradation of ubiquitinated cargo. Both the UPS and autophagy play complementary roles in the degradation of misfolded protein aggregates such as α-synuclein [2,151–152,84,86]. Heavily oxidized stable protein aggregates are not suitable for proteasomal degradation, and autophagy is thought to also play a major role in the degradation of oxidized protein aggregates [19–20]. The UPS and autophagy are complementary pathways, where alterations in the rate of one system are reported to modify those of the other one. In particular, impairment of the UPS system triggers autophagy [82–83,153]. Interestingly, while contradicting results exist regarding the ability of proteasome inhibitors to induce selective neurodegeneration [154–159], impairment of autophagy seems to selectively induce dopaminergic cell loss *in vivo* [12–13,160]. Indeed we observed that MG132 induces autophagy. However, whereas inhibition of ATG5-dependent autophagy stimulated PQ and MPP^+ toxicity no additional effect was induced by proteasome inhibition. The fact that inhibition of autophagy stimulates PQ and MPP^+ toxicity without exerting an effect on the accumulation of Ub-bound proteins (this work and [33]), suggests that the protective effects of autophagy are independent from its role in protein degradation.

Ubiquitinated proteins are selectively targeted to the autophagosome-lysosome system via Ub binding proteins, primarily p62/SQSTM [99,10]. p62 has been found in LBs from PD and dementia with LBs (DLB) brains [161–164], neurofibrillary tangles from Alzheimer's disease brains, and in Huntingtin aggregates [4,165–166]. Likewise, it has been shown that α-synuclein inclusions and oxidized proteins can be degraded through the p62-dependent autophagy clearance [164,21]. A recent report demonstrated that the UBA domain, which has a low affinity for Ub, also mediates the formation of inactive p62 dimmers, and that p62 dimerization and Ub-binding are mutually exclusives [74]. In our study, we found that the inhibition of Ub-activating enzymes (E1s) and the depletion of the Ub-protein pool induced by PQ and MPP^+ are paralleled by p62 inactivation/dimerization (Fig. 8). As we reported before [33], PQ and MPP^+ induce a dose- and time-dependent impairment in autophagy flux. Thus, our results suggest that p62 inactivation by Ub-protein depletion might be a mechanism by which PQ and MPP^+ also impair autophagy (Fig. 8). p62 has been reported to regulate mitophagy and stress response signaling via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) among others [14,74,167–169]. It is likely that p62 dimerization might be regulating the stress response of cells treated with PQ and MPP^+, and we expect to address this in the future.

Clearance of misfolded/aggregated α-synuclein has been shown to be mediated by both autophagy and the ubiquitin/proteasome pathways [86,84–85]. Ubiquitination of α-synuclein by E3 Ub-ligases (seven in absentia homologue-1 [SIAH1], E6 associated protein

[E6-AP], neural precursor cell expressed developmentally down-regulated protein 4 [Nedd4], tumor necrosis factor-receptor associated factor 6 [TRAF6]) has been proposed to regulate its degradation via the proteasome or endosomal-lysosomal degradation pathways [170–174].Interestingly, Ub-independent α-synuclein degradation via the 20S proteasome has also been reported [175]. Recent studies have demonstrated that ubiquitination of α-synuclein in different lysine residues mediates diverse effects including the formation of protein inclusions or Lewy bodies [176–178], which is now considered a protective mechanism against the accumulation of toxic protofibrillar intermediates, as aggregation of α-synuclein might be required for its detoxification [179]. PQ alone had no effect on the endogenous α-synuclein levels, but when WT or mutant A53T α-synuclein were overexpressed, PQ induced an increase accumulation of α-synuclein in its monomeric form. Indeed, ubiquitination of monomeric and fibrillar α-synuclein has been reported previously [180]. Furthermore, we observed that in yeast cells, where α-synuclein is localized at the plasma membrane, PQ treatment induced the accumulation of membrane foci, suggesting that alterations in protein ubiquitination might alter membrane sorting or turnover of α-synuclein. In fact it has been demonstrated that in yeast, localization of α-synuclein to the plasma membrane requires the secretory pathway [181], and α-synuclein was also reported to be ubiquitinated by Nedd4 [170], a homologous to the E6-AP carboxyl terminus [HECT]-domain E3 that functions at the plasma membrane in the turnover/sorting of a number of membrane-associated proteins [182].

Overall, our results uncover a new mechanism by which environmental (PQ) and mitochondrial toxicants (MPP^+) impair Ub-dependent proteostatic mechanisms. Post-transcriptional depletion of the Ub-protein pool impairs both proteasome and p62-autophagy mediated protein degradation pathways. Depletion of the Ub protein pool seems to be associated with the accumulation of oxidized proteins, alterations in the levels/distribution of α-synuclein, and precede the impairment in proteasome activity and autophagy flux (Fig. 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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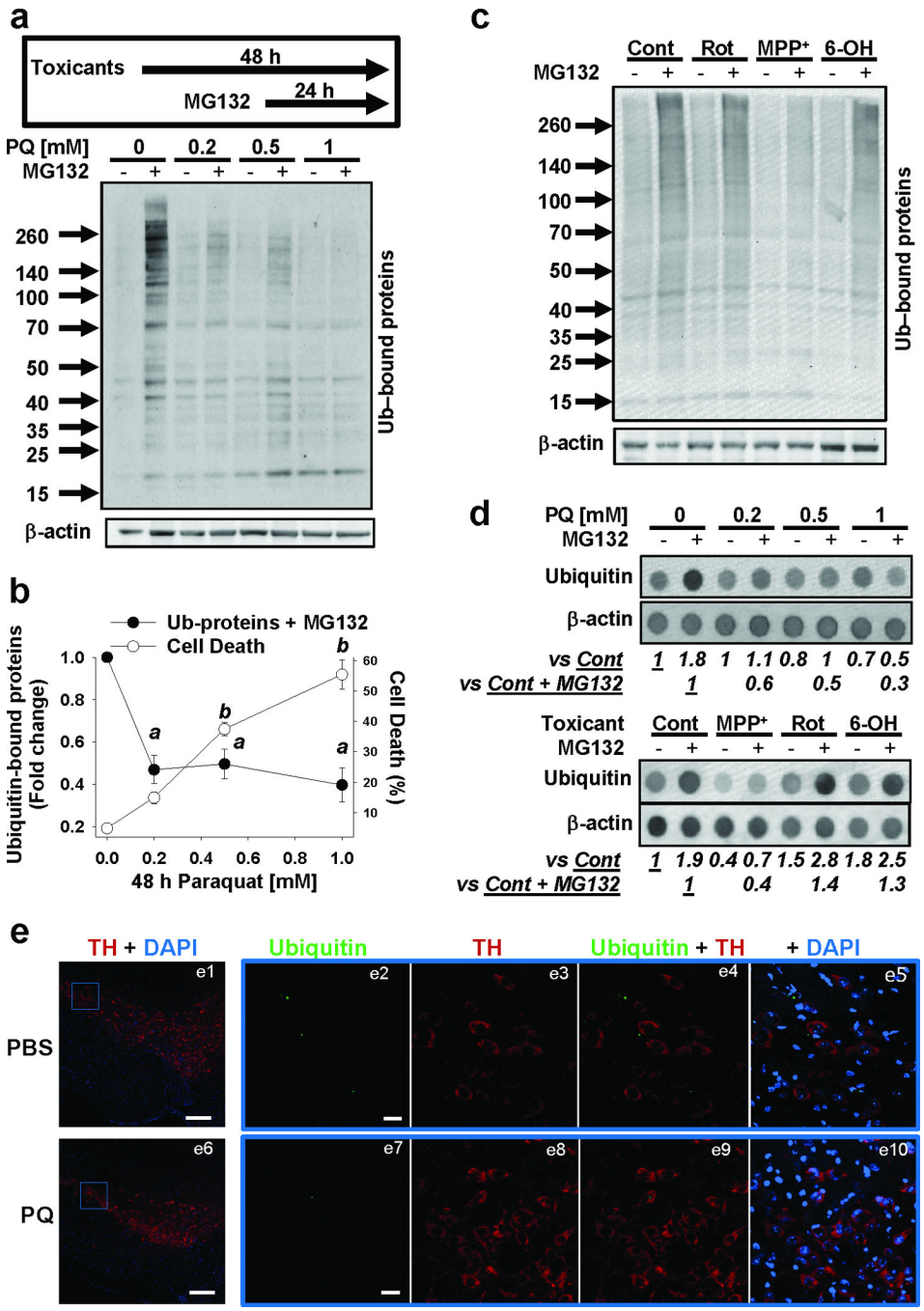

Fig. 1. PQ and MPP$^+$ impair protein ubiquitination

a. Ub–bound proteins were evaluated by WB in SK-N-SH cells treated with PQ for 48 h. Cells were treated with the proteasome inhibitor MG132 (0.2 μM) 24 h prior to analysis. **b**. Relative quantification (densitometry) of Ub-bound proteins in the presence of MG132 (●). Cells were treated as explained before (**a**). Data was normalized to β-actin and represented as fold change with respect to control (no PQ treatment). Cell death (○) was determined by the loss of plasma membrane integrity (PI uptake) and represented as % of cells with high PI fluorescence. Data are means ± S.E of at least n = 3. One-way ANOVA, Holm-Sidak *post*

hoc test: *a*, p<0.05; Kruskal-Wallis One Way ANOVA on Ranks, Dunn's *post hoc* test: *b*, p<0.05 compared to the corresponding control (no drug treatment). **c**. Changes in the levels of Ub-bound proteins were determined by WB in cells treated for 48 h with the complex I inhibitors MPP^+ (2.5 mM) and rotenone (Rot, 4 μM) and the hydroxylated dopamine analog 6-OHDA (6-OH, 50 μM). **d**. Cells were treated as explained above (**a** and **c**) and ubiquitinated proteins and aggregates were evaluated by dot blot. Relative quantification of ubiquitinated proteins (*numbers in italics*) was normalized to β-actin and represented with respect to the indicated control. **e**. Immunohistochemistry detection of ubiquitinated proteins in the substantia nigra of C57Bl/6 mice treated for 9 weeks with PBS (e1-e5) or PQ (e6-e10). Blue squares in e1 or e6 depict the area of magnification for e2-e5 (PBS) or e7-e10 (PQ) panels. Scale bars, e1 and e6: 200 μm; e2-e5 and e7-e10: 20 μm. TH+, tyrosine hydroxylase positive neurons.

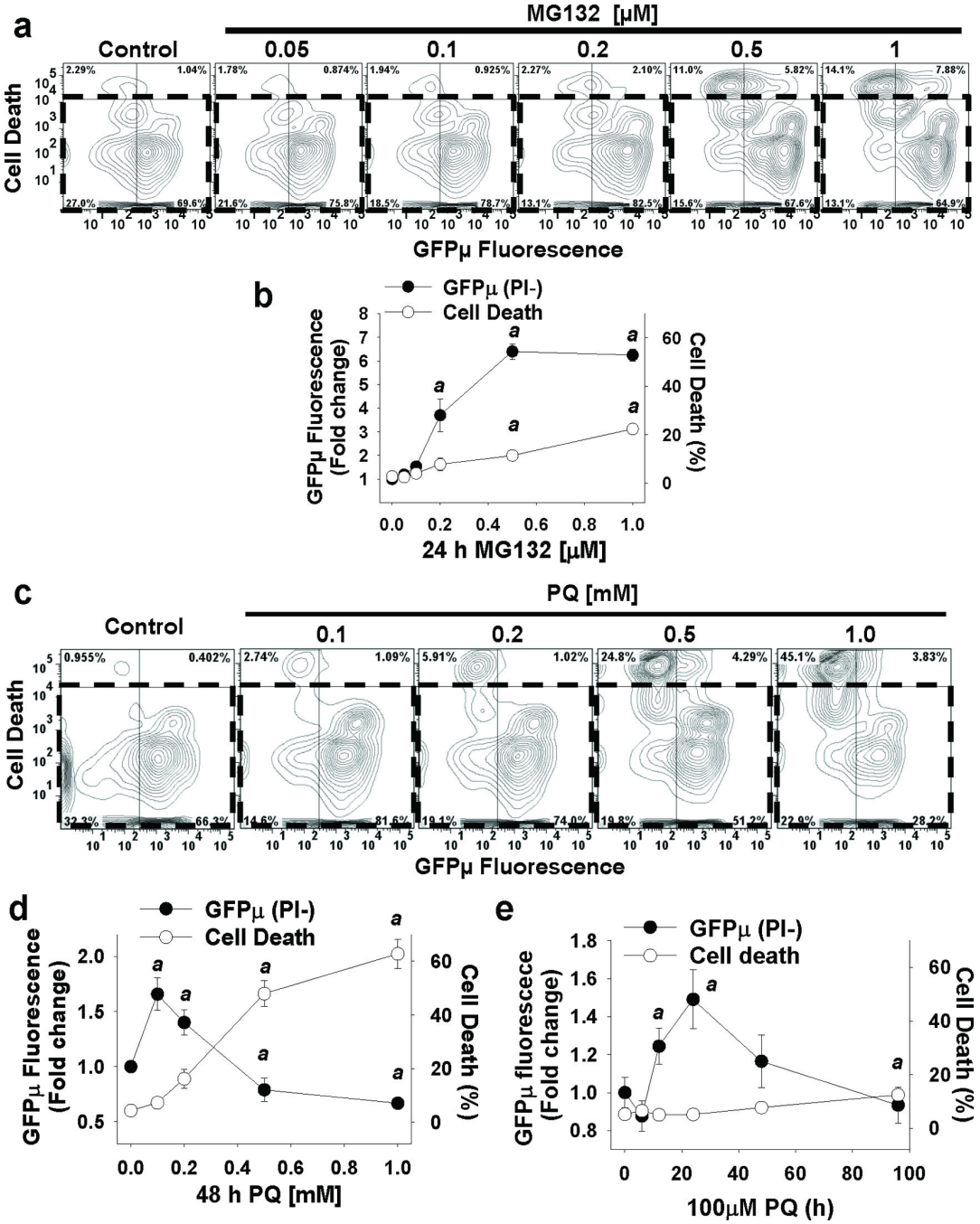

Fig. 2. PQ induces a transient accumulation of GFPμ, a reporter for the UPS

In **a** and **c**, the simultaneous analysis of cell death and changes in the levels of GFPμ induced by MG132 (24 h) or PQ treatment (48 h) was done by flow cytometry. Data are represented in two-dimensional 5% probability contour plots of changes in PI uptake (y axis) *vs* changes in GFPμ fluorescence (x axis). Broken squares depict how viable cells were selected. %s in contour plots represent the number of cells per quadrant. Using this type of analysis (gating), the changes in GFPμ induced by MG132 or PQ were quantified in **b, d** and **e** graphs. The geometric mean of GFPμ fluorescence intensity (●) was assessed in viable cells (PI negative

[-]) and represented as fold change with respect to control. Cell death (○) was represented as % of cells with high PI fluorescence. Data in graphs are means ± S.E of at least n = 3. Kruskal-Wallis One Way ANOVA on Ranks, Student-Newman-Keuls (SNK) *post hoc* test: *a*, $p<0.05$ compared to the corresponding control (no drug treatment).

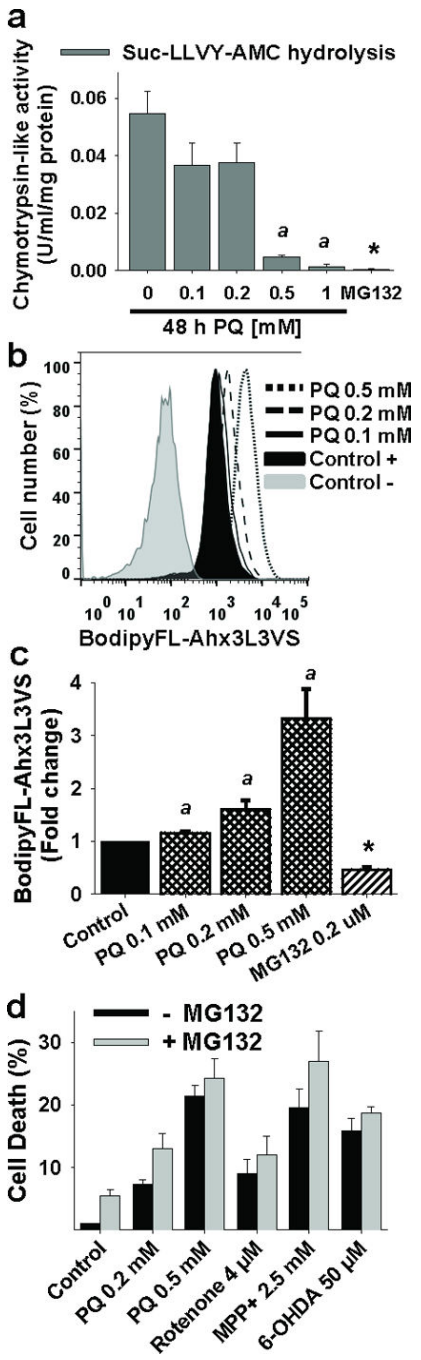

Fig. 3. Effects of PQ and MPP$^+$ on the activity of the proteasome

Cells were treated with the indicated toxicants for 48 h. **a**. The proteasomal 20S chymotripsyn–like activity was evaluated in total cell lysates by evaluation of the changes in AMC fluorescence released from the hydrolysis of Suc-LLVY-AMC. The proteasome inhibitor MG132 was used as a positive control (5 μM for 6 h). Data are expressed as U/ml/mg protein. **b**. Changes in the activity of the proteasome were evaluated in live cells using the cell permeable proteasome substrate BodipyFL-Ahx3L3VS. Histograms represent the distribution of cells with different levels of BodipyFL-Ahx3L3VS fluorescence. **c**. The

geometric mean of BodipyFL-Ahx3L3VS fluorescence intensity was quantified and represented as fold change with respect to control. MG132 was used as a positive control (0.2 μM for 16 h prior to the incubation with BodipyFL-Ahx3L3VS) . Background fluorescence (Control- in **b**) was subtracted. **d**. Cell death was determined in the presence or absence of MG132 (0.2 μM) by evaluating the loss of plasma membrane integrity (PI uptake) and represented as % of cells with high PI fluorescence. Data in graphs are means ± S.E of n = 3–5. Kruskal Wallis One-way ANOVA on ranks, Student Newman Keuls *post hoc* test: *a*, $p<0.05$; Mann Whitney Rank Sum t-test: *$p<0.05$ compared to the corresponding control (no drug treatment).

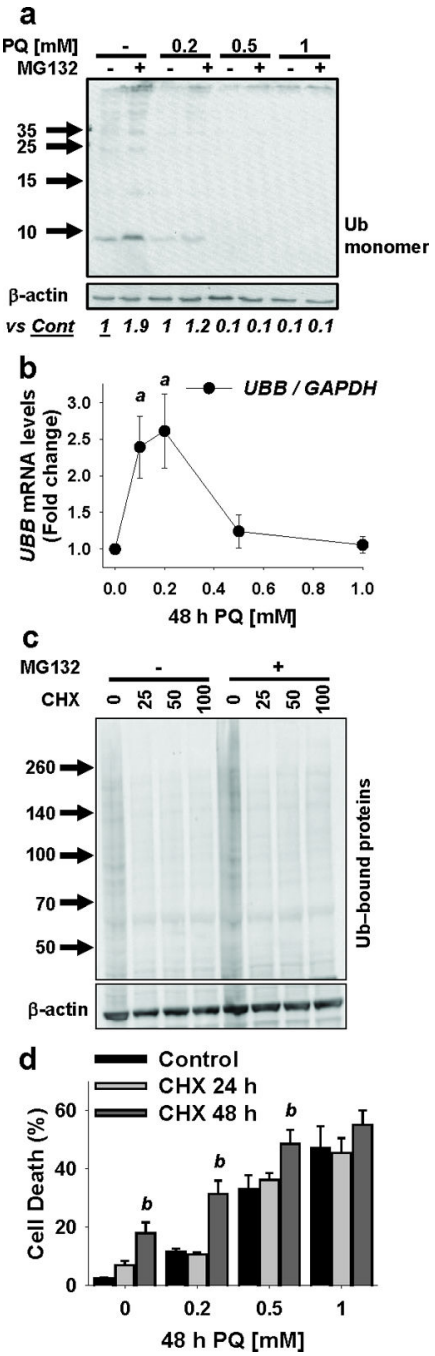

Fig. 4. PQ impairs Ub-protein synthesis

a. Changes in the levels of Ub–monomers and free chains were evaluated by WB (18% Tris glycine gels) in lysates from SK-N-SH cells treated with PQ for 48 h. Cells were treated with the proteasome inhibitor MG132 (0.2 μM) 24 h prior to analysis. Relative quantification of ubiquitinated proteins (*numbers in italics*) was normalized to β-actin and represented with respect to control. **b**. RT-PCR analysis of the changes in poly-ubiquitin gene *UBB* mRNA levels in cells treated with PQ for 48 h. Data were normalized using *GAPDH* as endogenous housekeeping gene. Results are expressed as changes in the relative

expression level compared to the control (no PQ treatment). **c**. Ub–bound proteins were evaluated by WB in cells treated with CHX for 48 h in the presence or absence of the proteasome inhibitor MG132 (as explained in **a**). **d**. Cell death was determined in cells treated with PQ, in the presence or absence of CHX (100 μM) by evaluating the loss of plasma membrane integrity (PI uptake) and represented as % of cells with high PI fluorescence. Data in graphs are means ± S.E of n = 3–5. One-way ANOVA, Holm-Sidak *post hoc* test: *a*, $p<0.05$ compared to the corresponding control (no drug treatment). Two-way ANOVA, Holm-Sidak *post hoc* test: *b*, $p<0.05$ compared to the corresponding PQ concentration without CHX treatment.

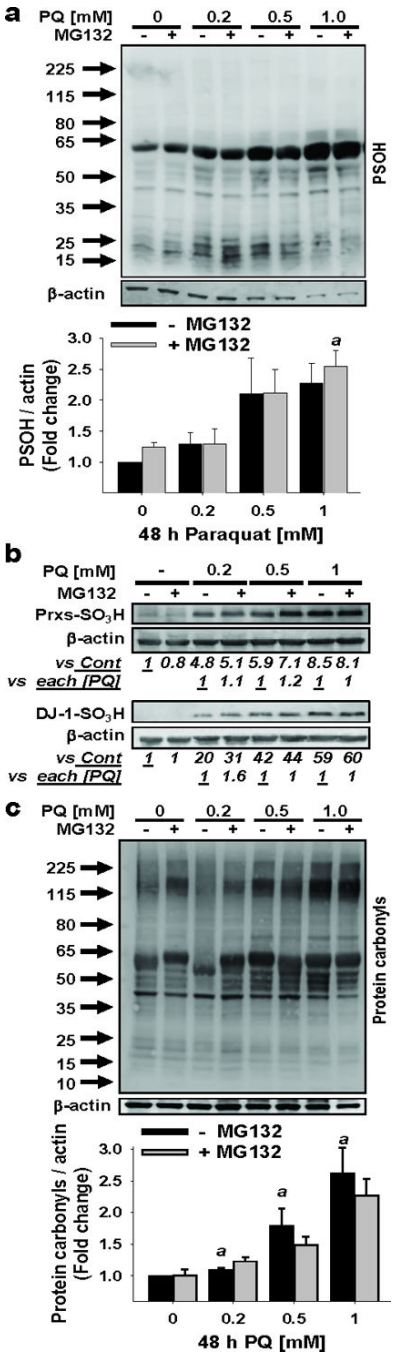

Fig. 5. The accumulation of oxidized protein induced by PQ is not regulated by the proteasome

Cells were treated with PQ for 48 h in the presence or absence of 0.2 μM MG132 (as exemplified in Fig. 1a). **a**. PSOHs were determined in cells incubated with the PSOH selective probe dimedone prior to the analysis. PSOHs were visualized using the anti-PSOH modified Cysteine 2-Thiodimedone-specific antibody. **b**. Levels of irreversibly oxidized DJ-1 and Prxs (PSO_3H) were detected by WB. Relative quantification of ubiquitinated proteins (*numbers in italics*) was normalized to β-actin and represented with respect to the indicated control. **c**. Protein carbonyls were detected by WB in total cell lysates derivatized

with DNPH. Carbonylated proteins were detected using anti-DNP antibody. Graphs indicate the densitometry analysis of changes in the levels of PSOHs or protein carbonyls normalized to β-actin and expressed as fold change with respect to control. Data are means ± S.E of five independent experiments. Kruskal-Wallis One Way ANOVA on Ranks, Student-Newman-Keuls (SNK) *post hoc* test: *a*, $p<0.05$ compared to the corresponding control (no drug treatment).

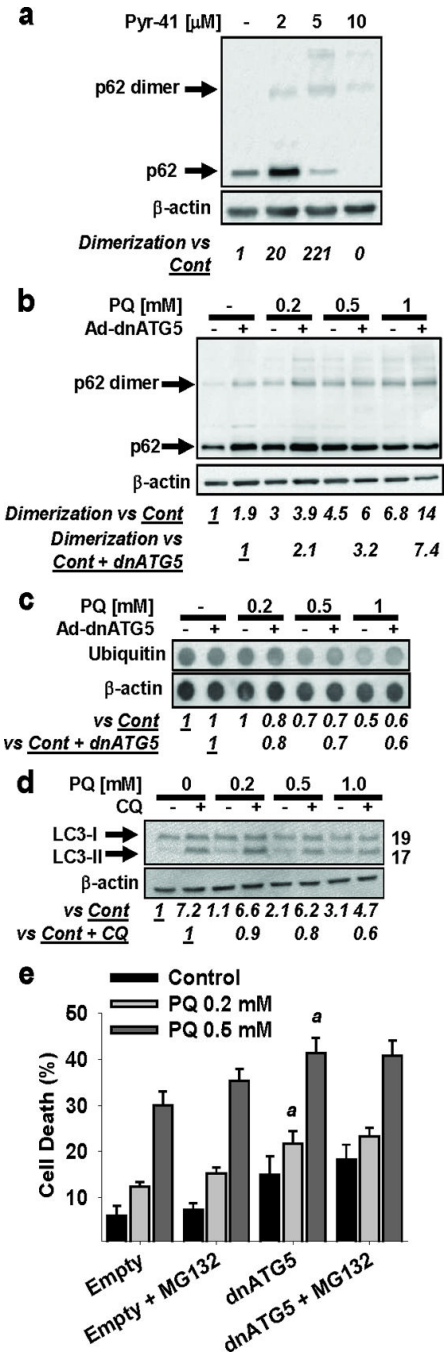

Fig. 6. PQ treatment induces the dimerization/inactivation of p62 and impairs autophagy

a. Cells were treated with the Ub-conjugating enzyme inhibitor (E1) Pyr-41 for 24h. p62 dimerization (inactivation) was evaluated by WB. Dimerization of p62 is evidenced by an increase in p62 dimers with the concomitant decrease p62 monomers. **b**. p62 dimerization was also evaluated in cells treated with PQ for 48h. When indicated, cells were transduced with dnATG5 or Empty viruses (-dnATG5) 24 h before PQ treatment. Relative quantification of p62 dimerization was represented as p62 dimers / p62 monomers ratio, normalized to β-actin and expressed with respect to the indicated control (*numbers in italics* in **a** and **b**). **c**.

Cells were treated as explained above (**b**) and ubiquitinated proteins and aggregates were evaluated by dot blot. Relative densitometry quantification of ubiquitinated proteins was normalized to β-actin and represented with respect to the indicated control (*numbers in italics*). **d**. Alterations in autophagy flux induced by PQ were determined by changes in the levels of the autophagosome marker LC3-II in the presence of CQ (40 μM, incubated 4 h prior to analysis), an inhibitor of lysosomal cargo degradation. Relative densitometry quantification of LC3-II (*numbers in italics*) was normalized to β-actin and represented with respect to the indicated control. **e**. Cell death was determined in cells treated with PQ, in the presence or absence of MG132 (0.2 μM). When indicated, cells were transduced with dnATG5 or Empty (- dnATG5) viruses 24 h before PQ treatment. Cell death was evaluated by the loss of plasma membrane integrity (PI uptake) and represented as % of cells with high PI fluorescence. Data are means ± S.E of n = 3–5. Two-way ANOVA, Holm-Sidak *post hoc* test analysis of data without MG132: *a*, $p<0.05$ dnATG5 *vs* Empty within the corresponding PQ category.

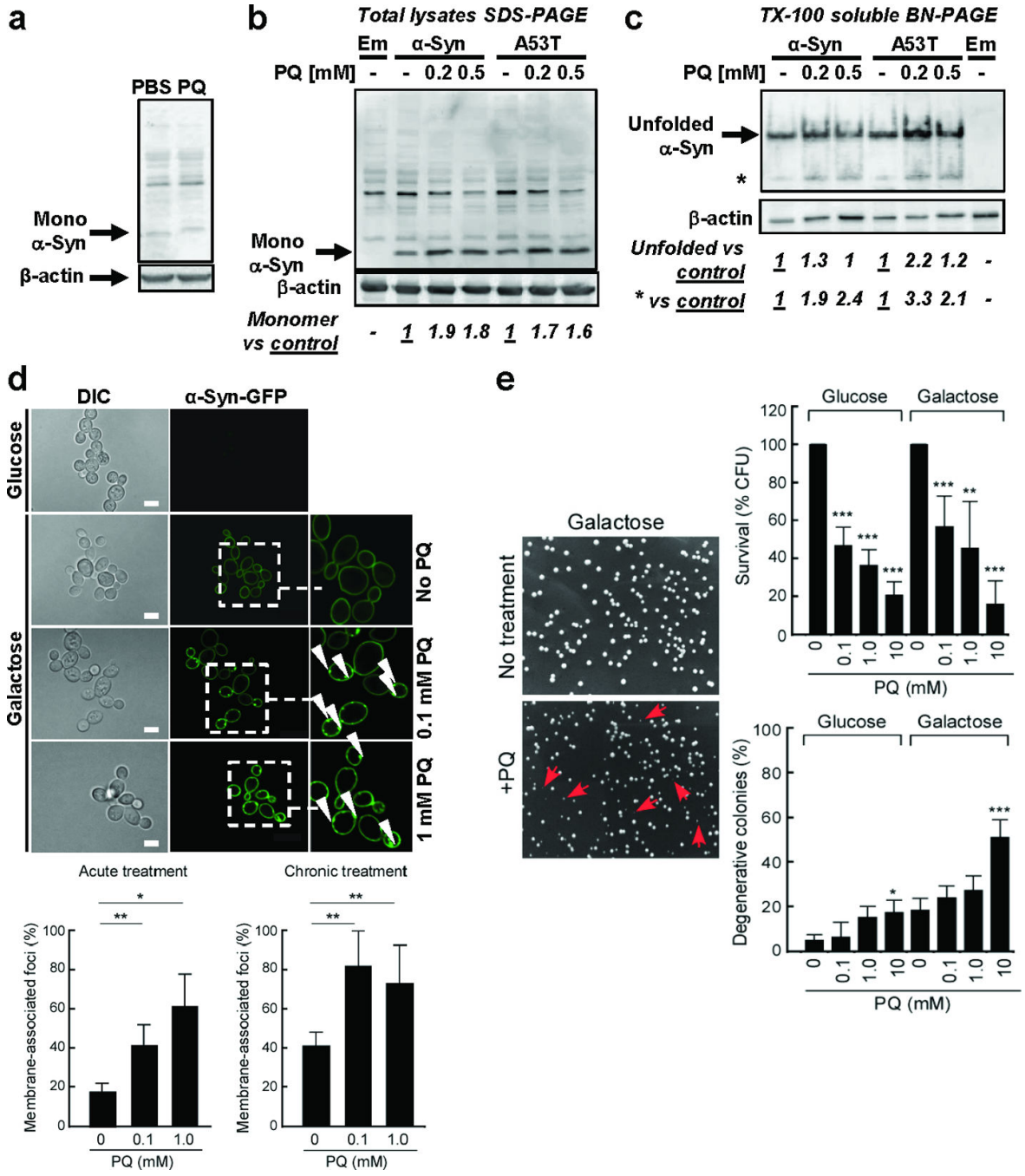

Figure 7. Effect of PQ on α-synuclein accumulation and distribution

a. WB analysis of α-synuclein levels in the midbrain of C57Bl/6 mice treated for 9 weeks with PQ or PBS. **b-c**. Cells were transduced with Ad-Empty, Ad-α-synuclein or Ad-A53T for 24h (3 MOI), washed and then treated with or without PQ (48 h). Whole cell lysates or TX-100 insoluble fractions were analyzed by SDS (**b**) or BN--PAGE (**c**), respectively, and α-synuclein was visualized by WB. Numbers (*italics*) represent the densitometry analysis normalized to β-actin with respect to the corresponding control. **d**. Wild-type *S. cerevisiae* cells containing genome-integrated human α-synuclein-GFP expression cassette under the

control of *GAL1* promoter were cultured in the medium containing 2% glucose or 2% galactose. The galactose-grown cells were then treated with the indicated amounts of PQ for 1 h (acute treatment), 48 h (chronic treatment). Subcellular distribution of α-synuclein-GFP was visualized by confocal microscopy. Shown are representative images of cells that have been acutely treated with PQ. Scale bars are 5 μm. Arrows indicate membrane associated foci of α-synuclein-GFP. Bar graphs show quantitation of α-synuclein-GFP foci. At least 300 cells per condition, per sample were analyzed. **e**. Yeast cultures described above were diluted to 300 cells and plated for survival on glucose- or galactose-containing plates with or without PQ. Following 4-days incubation at 28°C, the plates were inspected for colony forming units and the presence of small, degenerative colonies. The *left panel* shows representative images of cell growth on galactose plates with and without 1 mM PQ. Arrows indicate degenerative colonies. The *panels on the right* show quantitation of cell survival and percentage of degenerative colonies on glucose and galactose-containing plates containing the indicated amounts of PQ. Bar graphs are means ± S.D. (n=4); *p<0.05, **p<0.01, ***p<0.001 by unpaired *t*-test.

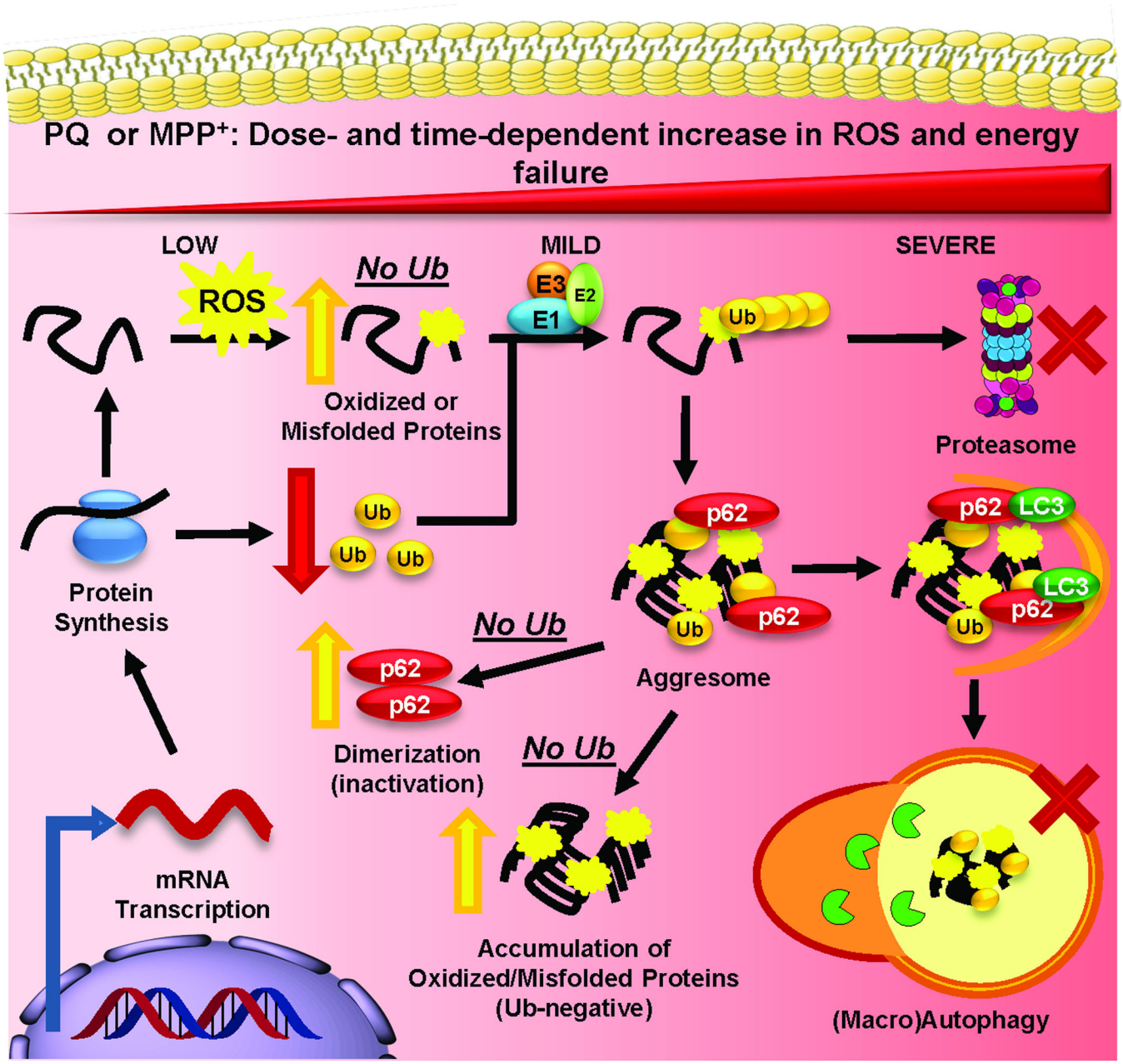

Fig. 8. PQ- and MPP$^+$-induced Ub-protein depletion and p62 inactivation are early steps in the impairment of Ub-dependent protein degradation pathways (UPS and autophagy)

Our data suggests that PD-related toxicants PQ and MPP$^+$ deplete the Ub protein pool (red arrow) by a mechanism that might involve oxidative mRNA damage, energy failure, or altered Ub protein stability. Ub depletion leads to the inactivation (dimerization) of the ubiquitin binding receptor p62 that directs ubiquitinated cargo for degradation via the authophagosome-lysosome pathway. Ub-protein depletion and p62 inactivation impair Ub-dependent protein degradation pathways, and parallel the accumulation of oxidized/misfolded proteins (yellow arrows) and alterations in the levels/distribution of α-synuclein (not exemplified). Severe/chronic oxidative stress and/or energy failure induced by

environmental and mitochondrial toxicants would eventually lead to a decrease in the activity of the proteasome and impaired autophagy flux (red crosses).