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LRRK2, but not pathogenic mutants, protects against H_2O_2 stress depending on mitochondrial function and endocytosis in a yeast model

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ABSTRACT

Background: Mutations in *LRRK2* are the most common genetic cause of Parkinson's disease (PD). Studies in the yeast *Saccharomyces cerevisiae* have provided valuable insights into the mechanisms of cellular dysfunction associated with the expression of faulty PD genes.
Methods: We developed a yeast model for full-length LRRK2 studies. We expressed wild-type (wt) LRRK2 and mutations and evaluated their role during oxidative stress conditions. The involvement of mitochondria was assessed by using rho-zero mutants and by evaluating reactive oxygen species (ROS) production and mitochondrial membrane potential by flow cytometry. The involvement of endocytosis was also studied by testing several endocytic mutants and by following the vacuolar delivery of the probe FM4-64.
Results: Expression of LRRK2 in yeast was associated to increased hydrogen peroxide resistance. This phenotype, which was dependent on mitochondrial function, was not observed for PD-mutants G2019S and R1441C or in the absence of the kinase activity and the WD40 repeat domain. Expression of the pathogenic mutants stimulated ROS production and increased mitochondrial membrane potential. For the PD-mutants, but not for wild-type LRRK2, endocytic defects were also observed. Additionally, several endocytic proteins were required for LRRK2-mediated protection against hydrogen peroxide.
Conclusions: Our results indicate that LRRK2 confers cellular protection during oxidative stress depending on mitochondrial function and endocytosis.
General significance: Both the loss of capacity of LRRK2 pathogenic mutants to protect against oxidative stress and their enhancement of dysfunction may be important for the development of PD during the aging process.
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1. Introduction

Parkinson's disease (PD) is a fatal neurodegenerative disorder of the central nervous system. Most of the PD cases are sporadic, although rare familial forms of the disease have been linked to mutations in several genes, providing research opportunities for pathogenic mechanisms [1].

Despite intensive research, the cause of PD remains obscure. Age is apparently the greatest risk factor and many studies have implicated mitochondrial dysfunction, oxidative stress, protein quality control, autophagy and vesicular trafficking in PD pathogenesis or progression [2]. Concerning mitochondrial dysfunction, extensive evidence has indicated a crucial role of this event in the pathogenesis of PD. Particularly, it was shown that mitochondrial toxins can cause Parkinsonism, and that the activity of mitochondrial complex I is decreased in PD patients [3]. Also, several genes associated to familial PD are involved in mitochondrial function [4].

The most important genetic cause of both familial and a significant proportion of apparently sporadic PD cases are autosomal-dominant mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene [1,5]. LRRK2 has also been tentatively implicated in mitochondria dysfunction and in the response to oxidative stress [6–9]. This is an attractive hypothesis as it suggests that different PD genes converge in a common mitochondrial pathway. Yet, LRRK2 is a large and complex protein, with multiple enzymatic and protein-interaction domains, whose biological function remains largely unknown. In the mammalian brain, LRRK2 is present throughout the cytoplasm associated to various membranes and vesicular structures [10]. Also, a small percentage of LRRK2 was found associated to the mitochondrial outer membrane [11]. This kinase has been implicated in several biological processes, including in the endocytosis of synaptic vesicles [12,13], autophagy regulation [14], and neurite outgrowth [15].

The LRRK2 domains include a leucine-rich repeat (LRR) domain, a Roc (Ras of complex protein) domain related to the Ras-related GTPase

Abbreviations: H_2O_2, hydrogen peroxide; PD, Parkinson's disease; ROS, reactive oxygen species; *S. cerevisiae*, *Saccharomyces cerevisiae*; wt, wild-type; GFP, green fluorescent protein; $\Delta\Psi m$, mitochondrial membrane potential

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superfamily, a COR (C-terminal of Roc) domain, a serine/threonine protein kinase domain, and a C-terminal WD40 repeats domain [16]. Although the presence of kinase and GTPase domains suggest a role of LRRK2 in cellular signaling, the existence of multiple protein–protein interaction domains, such as LRR, COR and WD40 repeats, suggests that LRRK2 may also have a role in the assembly of protein complexes [2,16]. The most common G2019S missense mutation lies in the kinase domain, but disease-causing *LRRK2* mutations are found almost throughout the protein [16].

Recently, relevant insights into the molecular mechanism of the PD-related protein α-synuclein have been provided by the yeast model system [17]. In fact, the study of human proteins in *Saccharomyces cerevisiae* is attractive due to the simple genetic manipulation, overexpression and deletion screens (reviewed in [18]). Here, we report the development of a yeast model of human LRRK2 expression to gain insights into the function and pathobiology associated with aberrant expression of this kinase.

2. Materials and methods

2.1. Vector construction for expression of wild-type (wt) and mutant forms of LRRK2 in yeast

Human wt *LRRK2* cDNA was obtained from a mammalian vector, generated and described in [11]. The LRRK2 construct was transferred to a yeast expression vector using the Gateway cloning system (Invitrogen) basically as reported [19]. The primers for cloning, listed in Table 1, were designed for the *LRRK2* gene from the published RNA sequence (GenBank: AY792511) with the AttB1 and AttB2 overhangs for recombination cloning according to the supplier's instructions. The PCR cycling conditions consisted of an initial denaturation at 94 °C for 2 min, 10 cycles each consisting of 92 °C for 10 s, 53 °C for 30 s, and 68 °C for 8 min, followed by 20 cycles each consisting of 92 °C for 10 s, 53 °C for 30 s, and 68 °C for 8 min + 10 s/cycle, with a final extension for 15 min at 68 °C. The directional gene transfer to the pAG423Gal-ccdB-GFP expression vector (Addgene plasmid 14197; [20] was performed via entry vector, using pDONR221 (Invitrogen) as donor plasmid, as described in the Gateway cloning protocol. For the construction of the ΔWD40 mutant, the PCR amplification was performed as for the wt LRRK2, but using a primer for the sequence just upstream of the WD40 domain (Table 1). A *LRRK2* containing the "triple kinase-dead" construct [21] in a Gateway-compatible vector was kindly provided by Dr. Mark Cookson and transferred to the pAG423Gal-ccdB-GFP vector via pDONR221. LRRK2 patient-based mutations (G2019S and R1441C) were introduced into pAG423Gal-LRRK2-GFP by PCR-based site-directed mutagenesis [22] using the oligonucleotides listed in Table 1. The PCR amplification was performed similarly as described before. PCR products were digested with DpnI and amplified in *Escherichia coli* NEB5a (New England Biolabs). All constructs were sequenced to ensure fidelity.

Table 1
Primers used in this study.

Purpose	Sequence
Cloning wt LRRK2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACC
	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCAACAGATGTTCGTCTCAT
Cloning LRRK2 ΔWD40	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACC
	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGGGCACAACCATATTCTT
Mutagenesis G2019S	CAAAGATTGCTGACTACAGCATTGCTCAGTACTGC
	GCAGTACTGAGCAATGCTGTAGTCAGCAATCTTTG
Mutagenesis R1441C	CTTGGCTCTTCAATATAAAGGCTTGCGCTTCTTCTTC
	GAAGAAGAAGCGCAAGCCTTTATATTGAAGAGCCAAG

2.2. Yeast strains, transformation and growth conditions

The *S. cerevisia*e strains used in this study are listed in Table 2. Cells depleted of mitochondrial DNA (ρ^0 cells) were generated from the W303 strain by a two-step growth on Yeast Peptone Dextrose (YPD; Difco) plates supplemented with ethidium bromide (40 μg/ml; Sigma) [23]. The different strains were transformed with the vectors encoding LRRK2 constructs/empty vector by the standard lithium acetate procedure [24]. Yeast cells were maintained and grown on selective minimal medium with 2% (w/v) glucose (Sigma), 0.67% (w/v) Bacto-yeast nitrogen base w/o amino acids (Difco), and the required amino acids. For induction of protein expression, cells grown in selective minimal media until exponential phase were diluted to 0.05 OD_{600} in selective induction media containing 0.05% (w/v) galactose (Sigma) plus 3% (w/v) glycerol, instead of glucose, and incubated at 30 °C with shake (200 rpm). For induction of protein expression in respiratory deficient cells, a mixture of 0.05% galactose and 2% raffinose (Acros Organics) was used.

All the experiments were performed with two independent clones of the LRRK2-GFP constructs (wt and mutants).

2.3. Western blot analysis

Preparation of protein samples, SDS-PAGE and Western blots were performed as previously described [25]. The primary antibodies, mouse monoclonal against GFP (1:2500; Clontech) and yeast phosphoglycerate kinase (Pgk1p; 1:6000; Molecular Probes), were used followed by an anti-mouse horseradish-peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) and revealed by chemiluminescence (ECL, Amersham). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat).

2.4. Viability assays

For oxidative stress experiments, exponential cultures (approximately 0.5 OD_{600}) were treated with 1–5 mM H_2O_2 for 1 h with shaking at 30 °C. Viability was assessed by colony-forming unit (CFU) counts after 2 days incubation at 30 °C on Sabouraud Dextrose Agar (Difco) plates, and expressed as percentage of time zero.

2.5. Endocytosis assay

Endocytosis was assessed using FM4-64 (N-(3-thiethylammonium-propyl)-4-(p-diethyl-aminophenylhexatrienyl) pyridinium dibromide) (Molecular Probes), basically as described [26]. Briefly, cells were incubated with 3 μg/ml FM4-64 along time at 30 °C and observed under an Eclipse E400 fluorescence microscope (Nikon). Images were captured with a *Digital Sight Camera System* (Nikon DS-5Mc) carrying built-in software for image acquisition (Nikon ACT-2U) and a semi-quantitative evaluation of cells with endocytic defects (lacking vacuolar staining) was

Table 2
Saccharomyces cerevisiae strains used in this study.

W303-1B	Mat*a ura3-1 leu2–3, 112 his3–11,15 trp1-1 ade2-1 can1–100*	Lab collection
BY4741	Mat *a*; *his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0*	EUROSCARF collection
ede1Δ	By4741; *YBL047c::kanMX4*	EUROSCARF collection
sla1Δ	By4741; *YBL007c::kanMX4*	EUROSCARF collection
end3Δ	By4741; *YNL084c::kanMX4*	EUROSCARF collection
end6Δ	By4741; *YCR009c::kanMX4*	EUROSCARF collection
bzz1Δ	By4741; *YKL129c::kanMX4*	EUROSCARF collection
vps21Δ	By4741; *YOR089c::kanMX4*	EUROSCARF collection
vps41Δ	By4741; *YDR080w::kanMX4*	EUROSCARF collection
ypt7Δ	By4741; *YML001w::kanMX4*	EUROSCARF collection

estimated by counting at least 300 cells per sample in three independent experiments.

2.6. Assessment of mitochondrial membrane potential (ΔΨm) and reactive oxygen species (ROS) production

To assess ΔΨm, 5×10^6 cells were incubated for 30 min at 30 °C with 40 nM of 3,3-dihexyloacarbocynine iodide ($DiOC_6$ (3)) (Molecular Probes). To assess intracellular superoxide anion production, cells were incubated with 5 μg/ml of dihydroethidium (DHE; Sigma) for 30 min at 30 °C. Sample analysis was performed in FACSCalibur (BD Biosciences) flow cytometer using CellQuest software (BD Biosciences). Twenty thousand cells were analyzed per sample.

3. Results

3.1. LRRK2 expression in yeast

To study human wt and mutant forms of LRRK2 in yeast, constructs were tagged with a C-terminal green fluorescent protein (GFP) and were expressed under the control of the regulatable *GAL10* promoter. Mutant forms of LRRK2 included the pathogenic mutations G2019S and R1441C, and a LRRK2 mutant containing three point mutations that disrupt kinase activity (3KD) [21]. A deletion in the WD40 domain required for LRRK2 function and neurotoxicity (ΔWD40) was also studied [27,28] (Fig. 1A).

Expression of wt and mutant forms of LRRK2-GFP in yeast was confirmed by Western analysis (Fig. 1B). As previously reported for native LRRK2 [29], a high amount of LRRK2-GFP fusion forms were found as insoluble protein (accumulated between the stacking and separating gel), while only a small fraction appeared as soluble protein (quantified in Fig. 1C). The insoluble protein may be due to the formation of LRRK2-GFP aggregates, as previously reported in several cell models for native LRRK2 [9,30–32]. In fact, by fluorescence microscopy, diffuse cytoplasmic fluorescence and variable size inclusions (indistinguishable for the distinct constructs) could be observed in yeast cells expressing the LRRK2-GFP constructs, in contrast to the control yeast that only exhibited cytoplasmic fluorescence (Fig. 1D). Because we were using an inducible *GAL* promoter, and the amount of galactose used correlated with expression levels, and consequent inclusion formation, we used a lower percentage of galactose (0.05%; supplemented with 3% glycerol) to minimize this effect.

As previously reported [29], no reproducible growth phenotype was obtained with the LRRK2-GFP constructs (Fig. 1E).

3.2. Wt LRRK2 is associated with protection against H_2O_2 toxicity

There has been extensive evidence linking oxidative stress to both the initiation and the progression of PD [33]. Several studies support a role for LRRK2 in the response to oxidative stress, usually mimicked by H_2O_2 exposure, but the outcome is controversial [7,34–37]. As such, our yeast model system was used to address this relevant issue. For

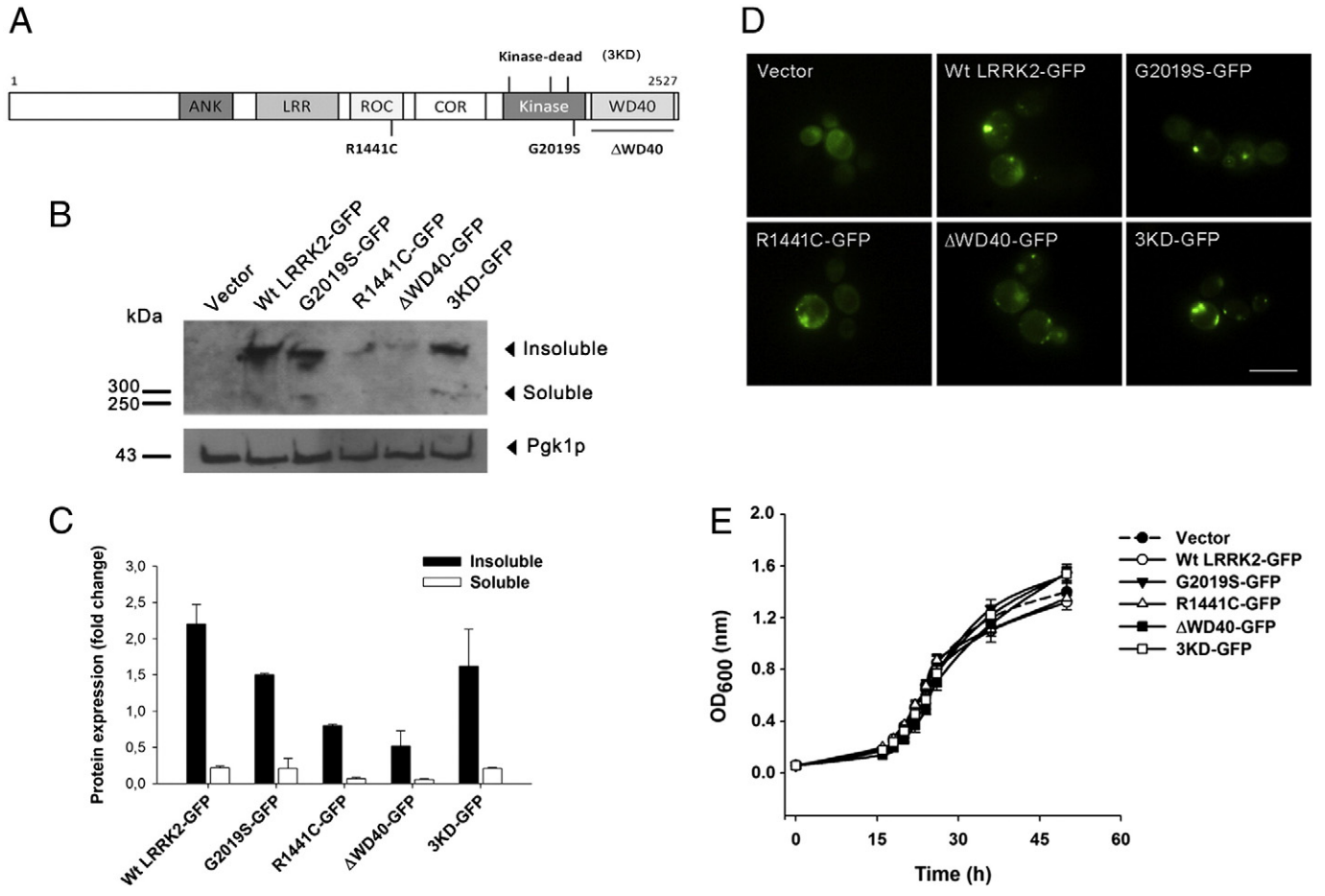

Fig. 1. Expression of human wt and mutant forms of LRRK2 in yeast. (A) Schematic of the domain structure of human LRRK2 with the studied mutations indicated. ANK, ankyrin; LRR, leucine-rich repeats; Roc, Ras of complex proteins; COR, C-terminal of Roc; and Kinase, mitogen-activated protein kinase kinase kinases. (B) Immunoblot analysis of cells expressing LRRK2-GFP constructs. Pgk1p was used as loading control. (C) Quantification of protein expression (insoluble and soluble fractions). Fold change was quantified relative to Pgk1p. (D) Fluorescence microscopy of yeast cells expressing GFP alone or LRRK2-GFP constructs. (E) Growth of control yeast (vector) and yeast expressing LRRK2 constructs in induction media along time. Yeast cell growth was assessed by optical density measurements at 600 nm. Values are means ± SE (n = 4). The effect on growth was not significant; ($P > 0.05$), two-way ANOVA.

that, cells expressing wt or mutant forms of LRRK2-GFP were exposed to H_2O_2 for 1 h. Expression of wt LRRK2-GFP resulted in a significant resistance to oxidative stress. In fact, in the presence of wt LRRK2-GFP, about 20% and 14% increase in the percentage of cell survival was obtained for 3 mM and 5 mM, respectively, when compared to the control yeast (Fig. 2A). On the contrary, for G2019S-GFP, R1441C-GFP, ΔWD40-GFP and 3KD-GFP no significant differences in the percentage of cell survival were observed (Fig. 2A). When 2% galactose was used to induce high levels of expression, the higher inclusion formation was associated to a loss of H_2O_2 resistance (not shown), supporting a role for soluble LRRK2 in this resistance.

Because in recent years a connection of LRRK2 with mitochondria has been suggested [2], we decided to determine whether functional mitochondria were determinant for LRRK2 response to oxidative stress in yeast. For that, a ρ^0 strain, which lacks mitochondrial genome, was used. Since the ρ^0 strains do not grow well in galactose alone, for induction of LRRK2 constructs expression, a combination of galactose and raffinose, a fermentable substrate, but not a mitochondrial function repressor, was used. We confirmed that, in this culture medium, the LRRK2-GFP resistance to H_2O_2 was not affected in the parental ρ^+ strain (Fig. 2B). Since ρ^0 cells are more sensitive to H_2O_2, a lower concentration of H_2O_2 (1 mM) was used in order to obtain a comparable percentage of cell death to the wt respiratory competent cells. In the absence of functional mitochondria, wt and mutant forms of LRRK2-GFP exhibited a similar percentage of cell survival to the control yeast (Fig. 2B). These results indicate an involvement of the mitochondrial pathway in the LRRK2 function. Based on this, markers of mitochondria dysfunction were checked in the wt strain expressing LRRK2-GFP constructs in response to H_2O_2, particularly ROS production and $\Delta\Psi m$. After 1 h treatment with 5 mM H_2O_2, a small decrease in ROS production for cells expressing wt LRRK2-GFP was observed. On the contrary, stimulation in ROS production was obtained for the mutants G2019S-GFP, R1441C-GFP and ΔWD40-GFP (higher for the two former pathogenic mutants). Interestingly, no effect on ROS production was observed for the 3KD mutant (Fig. 2C). The $\Delta\Psi m$ was also measured either in the absence and presence of 5 mM H_2O_2. For untreated cells, a decrease in $\Delta\Psi m$ was observed for all LRRK2-GFP constructs, though more evident for the pathogenic mutants G2019S-GFP and R1441C-GFP, when compared to the control vector (Fig. 2D; dark line). For H_2O_2-treated cells, though a typical depolarization was observed in the control vector, a hyperpolarization was obtained for the mutants G2019S-GFP and R1441C-GFP. For wt LRRK2-GFP, ΔWD40-GFP and 3KD-GFP, an intermediate response

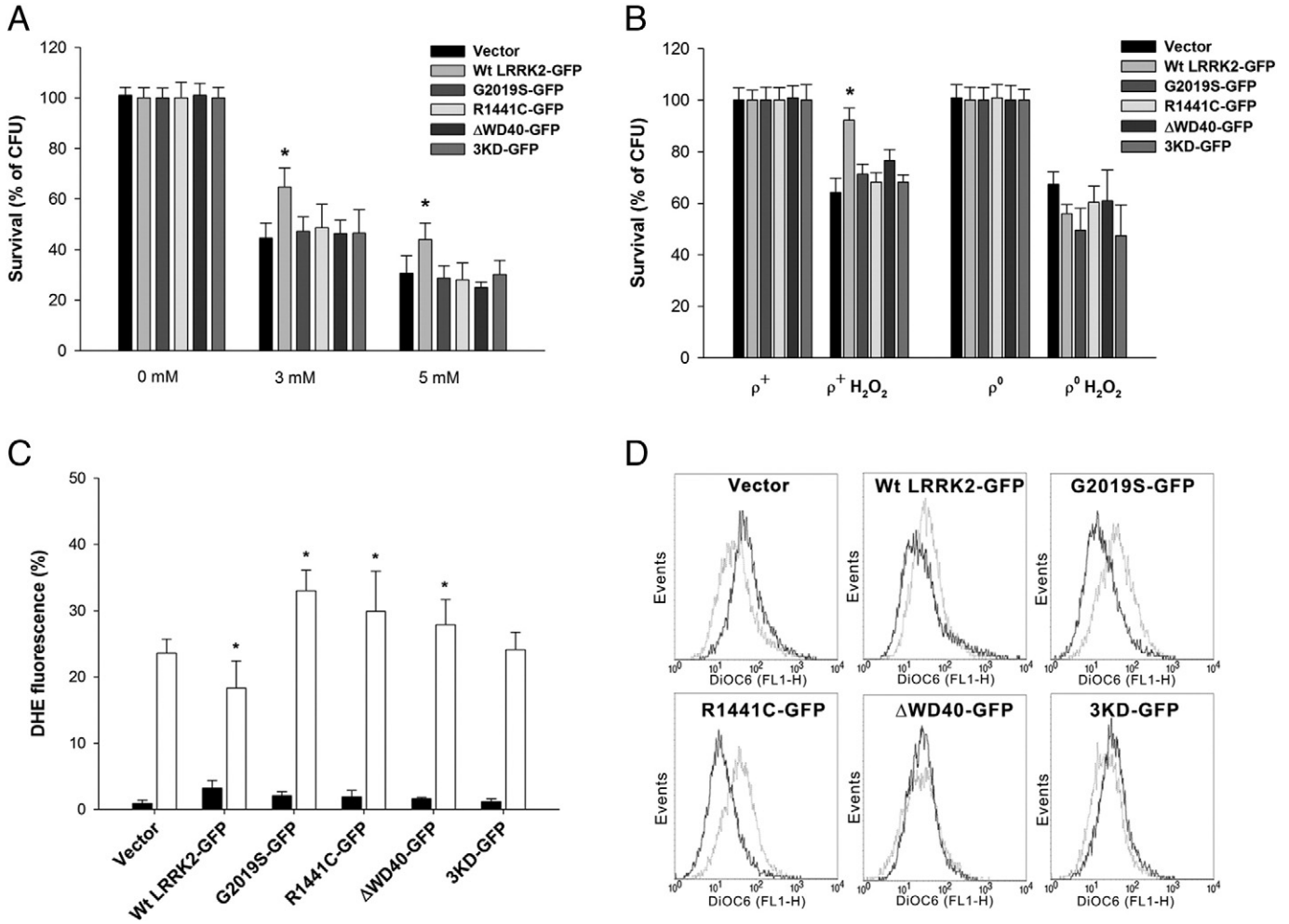

Fig. 2. Effect of wt and mutant forms of LRRK2 on cell survival upon H_2O_2 exposure. (A) Wt strain expressing LRRK2-GFP constructs or transformed with the empty vector (control) were grown until approximately 0.4 OD_{600} followed by treatment with the indicated concentration of H_2O_2 for 1 h. Data are mean ± SE (n = 4). *$P < 0.05$, by Student's *t*-test compared to vector. (B) Wt (ρ^+) and derived ρ^0 strains expressing LRRK2-GFP constructs or control were incubated in a combination of 0.05% galactose and 2% raffinose medium until approximately 0.4 OD_{600} followed by treatment with 1 mM H_2O_2 for 1 h. Cell survival was assessed by CFU counts; 100% survival corresponds to the number of CFU at time 0. Data are mean ± SE (n = 4). *$P < 0.05$, by Student's *t*-test compared to Vector. Cellular dysfunction associated to H_2O_2 treatment in yeast expressing wt or mutant forms of LRRK2. (C) ROS production was assessed by monitoring the increase in DHE fluorescence in untreated (black bars) or 5 mM H_2O_2-treated yeast cells (white bars) expressing LRRK2-GFP constructs and control yeast (vector) by flow cytometry. Data are mean ± SE (n = 4). *$P < 0.05$, by Student's *t*-test compared to vector. (D) Mitochondrial depolarization was assessed by monitoring the $DiOC_6(3)$ fluorescence change in untreated (dark line) and 5 mM H_2O_2-treated (light line) yeast cells expressing LRRK2-GFP constructs and control yeast (vector) by flow cytometry. Image shows monoparametric histograms of $DiOC_6(3)$ fluorescence representing one of the four independent experiments.

between the pathogenic mutants and the control vector was observed (Fig. 2D; light line). The ΔΨm experiments were also performed in ρ^0 cells without a functional respiratory chain. In these H_2O_2 treated cells, unlike for ρ^+ cells, the LRRK2 constructs exhibited a similar depolarization to the control vector (not shown). Together, these results suggest that the pathogenic mutants G2019S-GFP and R1441C-GFP further exacerbate the mitochondrial dysfunction in response to H_2O_2 toxicity. Moreover, since in ρ^0 cells the mitochondrial respiratory chain is not functional, these proteins may be acting on this complex.

3.3. LRRK2 function is dependent on the endocytic pathway

LRRK2 has been implicated in the modulation of synaptic vesicle trafficking [12,32]. The GTPase domain may be particularly important for this modulation since the expression of a LRRK2 fragment containing the GTPase domain was shown to impair endocytosis in yeast [29]. We decided to evaluate the effect of the full-length LRRK2 constructs on vesicular trafficking upon H_2O_2 treatment. For that, the internalization of the membrane-binding fluorescent dye FM4-64 was monitored after 2 h (when all the H_2O_2-untreated strains presented more than 90% vacuolar staining) incubation. As expected, in untreated cells (vector or LRRK2-GFP constructs), FM4-64 was rapidly endocytosed and accumulated at the vacuolar membrane (not shown). Upon treatment with 5 mM H_2O_2, a small percentage of control cells exhibited endocytic defects characterized by the appearance of dot like structures (Fig. 3A, green arrowhead; quantification in Fig. 3B and C) as described [38] in addition to the large ring-like vacuolar staining (Fig. 3A, white arrowhead). In cells expressing G2019S-GFP or R1441C-GFP, the percentage of cells with endocytic defects showed an increase of about 28% for G2019S-GFP and of 20% for R1441C-GFP, when compared to the control vector (Fig. 3B and C). In cells expressing wt LRRK2-GFP, ΔWD40-GFP or 3KD-GFP no major interference in endocytosis was observed, when compared to the control vector (Fig. 3B and C).

Since the endocytic process is genetically well defined in yeast, to assess the impact of vesicular trafficking on the cellular response to LRRK2, a panel of null yeast mutants in defined steps of the endocytic pathway was used (Fig. 3D). These included Ede1p (early immobile phase), End3p, Sla1p and End6p/Rvs161 (Mid/late immobile phase), Bzz1p (actin/mobile phase), Vps21p (vesicle transport), Vps41p and Ypt7 (transport from late endosomes to the vacuole) [12,39].

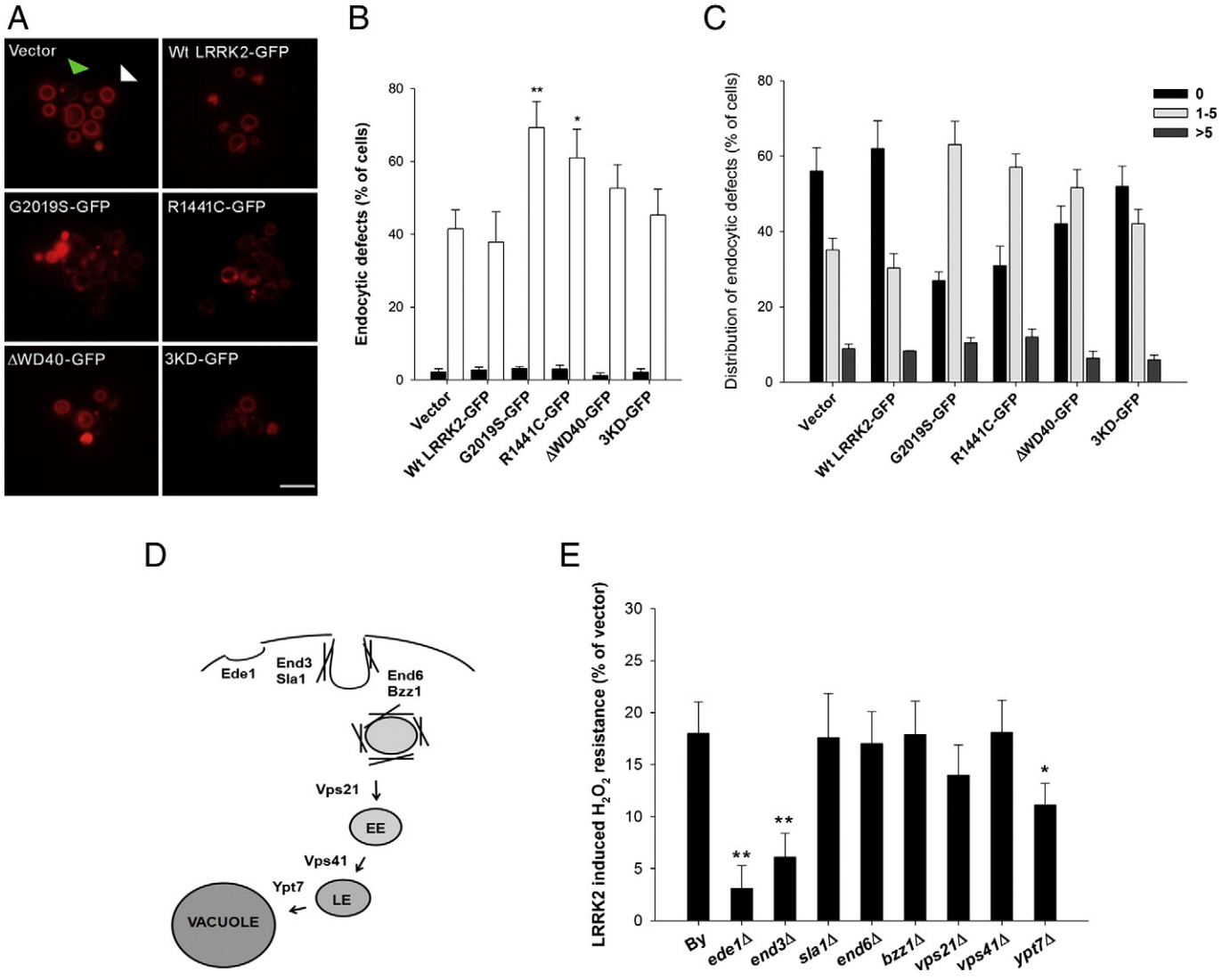

Fig. 3. Effect of LRRK2 on endocytosis during H_2O_2 treatment. (A) Representative photomicrographs of 5 mM H_2O_2-treated cells stained with FM4-64. Bar, 15 μm. White arrowhead indicates normal cell with vacuolar staining, and green arrowhead indicates a cell with endocytic defects. (B) Quantitative expression of endocytic defects (lacking vacuolar staining), untreated (black bars) or after 2 h incubation with 5 mM H_2O_2 (white bars). Values are mean ± SE (n = 3). (C) Distribution of endocytic defects (cells with different amounts of FM4-64-stained punctate structures). (D) Schematic of the tested endocytic mutants. (E) Stress resistance of endocytic mutant strains expressing wt LRRK2-GFP. Yeast cell growth was assessed by CFU counts after 1 h incubation with 5 mM H_2O_2 in induction medium. Data are mean ± SE (n = 4). $^*P < 0.05$, $^{**}P < 0.01$, paired *t*-test.

After treatment with H_2O_2, protection provided by wt LRRK2-GFP was strongly reduced when expressed in *ede1*Δ or *end3*Δ strains, and moderately reduced when expressed in *ypt7*Δ (Fig. 3E). In the remaining endocytic yeast mutants (*sla1*Δ, *bzz1*Δ, *vps41*Δ, *end6*Δ, *vps21*Δ), protection provided by wt LRRK2-GFP was similar as when expressed in the wt strain (Fig. 3E).

4. Discussion

In the present work, the yeast *S. cerevisiae* was used to study the full length LRRK2 protein. We observed, as reported before, that the overexpression of wt LRRK2 or PD-associated mutants G2019S and R1441C was not cytotoxic to yeast. However, unlike wt LRRK2, the PD-associated mutants were unable to protect cells against H_2O_2 toxicity. LRRK2 has been associated with the protection against mitochondrial stressors and other types of oxidative stress [7,34,36,37]. However, contradicting results, in which LRRK2 exacerbates stress, have also been reported [35,40]. Based on our observations, these contradicting results may be attributed to distinct experimental conditions. In fact, when a high percentage of galactose was used (2%), leading to higher expression levels of LRRK2, a small enhancement of H_2O_2 toxicity was also observed in our model (not shown). The protective function we observed when expressing LRRK2 at low levels, was in yeast, as reported for other cellular models [37,41], dependent on the kinase activity. This reinforces the importance of the LRRK2 catalytic domain in the resistance against stress, making the inhibition of LRRK2 kinase as a therapeutic approach against PD to be considered cautiously. Besides, in this work, the protective function of another LRRK2 domain, the WD40 repeats, was also investigated. Our results indicate that also this domain has a protective effect against H_2O_2-induced oxidative stress.

As reported for other cellular systems [42], also in yeast the LRRK2-mediated protection against H_2O_2-induced stress was lost for the pathogenic mutations G2019S and R1441C. This supports that deficits in oxidative stress resistance may be important in the development of PD.

The protective function of LRRK2 may be explained by a potential role in the homeostasis of oxidative stress within the cells, by acting on antioxidant proteins. In fact, this was suggested by the observation that LRRK2 mutants decrease the mitochondrial peroxidase peroxiredoxin 3 (PRDX3)[43] and the glutathione s-transferase P1 (GSTP1)[44] activity, contributing to mitochondrial dysfunction and increased oxidative damage. In accordance, we observed for the pathogenic mutants, in addition to the loss-of-function, an increase in ROS production and also an increase in endocytic defects, which may be a consequence of increased ROS. In cells with high energy demands, such as neurons, this enhancement of oxidative stress-induced dysfunction may be a relevant mechanism of pathogenicity. Oxidative stress is intimately linked with mitochondrial dysfunction. A role for mitochondria in LRRK2 function has already been suggested. Particularly, a small percentage of LRRK2 was found to be associated with the mitochondrial outer membrane [11], and it was shown that LRRK2 protects against mitochondrial stressors [6,42]. Additionally, it was recently reported that LRRK2 has a role in regulating mitochondrial dynamics [45,46] and $\Delta\Psi m$. In fact, it was observed both in PD patients [8] and in vitro [47] that the G2019S mutation reduces $\Delta\Psi m$. These reports are in accordance with our findings in yeast. The protection provided by LRRK2 in yeast was significantly decreased in cells lacking functional mitochondria. In addition, in this work, it was shown that G2019S and R1441C decreased $\Delta\Psi m$ in the absence of any stressor, which results in mitochondria hyperpolarization instead of depolarization upon H_2O_2-induced stress. This hyperpolarization may be due to a stimulation of the respiratory chain activity, since in ρ^0 cells this effect is lost. Because in ρ^0 yeast cells both respiration and mitochondrial-nuclear communication (e.g., retrograde signaling) are impaired [48], it will be interesting, in future work, to further ascertain the contribution of these processes for LRRK2 toxicity.

The stress protection offered by LRRK2 was significantly decreased in cells lacking several endocytic proteins. Taking into account the number of tested endocytic proteins that were able to affect the LRRK2-induced stress resistance, this pathway may have a major role in the LRRK2 function. Recently, it was reported that disease-associated LRRK2 interacts with members of the dynamin GTPase superfamily, proteins with a role in the regulation of membrane dynamics, important for both endocytosis and mitochondrial morphology, connecting these two processes [49]. Most of the proteins in the endocytic pathway are evolutionary conserved in mammals. Ypt7 is the homologue of Rab7, recently described to physically interact with LRRK2 in *Caenorhabditis elegans* [50] and Vps21 is the homologue of Rab5, described as a LRRK2 interactor, and suggested to co-regulate with LRRK2 the endocytic pathway [12]. In our model, however, only Ypt7 affected LRRK2 function in the cellular stress response. The two proteins with the most dramatic effects on wt LRRK2-mediated stress protection were Ede1p and End3p that play a role in the initial phase of vesicle formation [39]. Ede1 and End3 have homology to human Eps15, an adaptor protein involved in the epidermal growth factor (EGF) receptor (EGFR) endocytosis and trafficking. The Eps15 was the first identified substrate of Parkin-mediated monoubiquitination [51]. Therefore, it will be interesting to ascertain if Eps15 can also act as an LRRK2 substrate.

In conclusion, in the present work insights into the LRRK2 function and the pathogenic mechanisms of PD-associated mutations were provided. Particularly, it was shown that mitochondrial function and endocytosis seem to be intersecting pathways in the LRRK2 function. This yeast model may help to identify new associated/interacting proteins therefore contributing to a deeper understanding of the role of LRRK2 in PD. Besides its use for genetic screens, this model may also be adapted for chemical screening assays, based on the direct assessment of the yeast cell growth.

Acknowledgments

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