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Nrf2 mitigates LRRK2- and α-synuclein–induced neurodegeneration by modulating proteostasis

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Mutations in leucine-rich repeat kinase 2 (LRRK2) and α-synuclein lead to Parkinson's disease (PD). Disruption of protein homeostasis is an emerging theme in PD pathogenesis, making mechanisms to reduce the accumulation of misfolded proteins an attractive therapeutic strategy. We determined if activating nuclear factor erythroid 2-related factor (Nrf2), a potential therapeutic target for neurodegeneration, could reduce PD-associated neuron toxicity by modulating the protein homeostasis network. Using a longitudinal imaging platform, we visualized the metabolism and location of mutant LRRK2 and α-synuclein in living neurons at the single-cell level. Nrf2 reduced PD-associated protein toxicity by a cell-autonomous mechanism that was time-dependent. Furthermore, Nrf2 activated distinct mechanisms to handle different misfolded proteins. Nrf2 decreased steady-state levels of α-synuclein in part by increasing α-synuclein degradation. In contrast, Nrf2 sequestered misfolded diffuse LRRK2 into more insoluble and homogeneous inclusion bodies. By identifying the stress response strategies activated by Nrf2, we also highlight endogenous coping responses that might be therapeutically bolstered to treat PD.

Nrf2 | Parkinson's disease | LRRK2 | α-synuclein | proteostasis

Parkinson's disease (PD) is a progressively debilitating neurodegenerative condition that leads to motor and cognitive dysfunction. There are no disease-modifying therapies for this disease. Mutations in the genes encoding α-synuclein (1) and leucine-rich repeat kinase 2 (LRRK2) cause PD (2). Mutations in LRRK2 are the most common genetic cause of PD (3), and although mutations in α-synuclein are rare, abnormal α-synuclein accumulation and pathology characterize most PD cases (4). Furthermore, there is evidence of an epistatic relationship between LRRK2 and α-synuclein (5–7), suggesting that understanding how to block their ability to cause neurodegeneration might be beneficial across a wide swath of PD patients.

Mutations in both proteins have been implicated in protein misfolding and protein dyshomeostasis within the cell (8). Mechanisms to handle misfolded proteins more effectively within the cell include modulating endogenous cellular stress response pathways that up-regulate the protein degradation machinery or sequester the misfolded proteins into inclusion bodies (IBs) (9, 10). Nuclear factor erythroid 2-related factor (Nrf2) coordinates a whole program of gene expression that counters stress at multiple levels (11). Nrf2 was initially shown to bind to the antioxidant responsive element (ARE) and regulate the expression of detoxification genes (e.g., GST) (12) and oxidative stress-inducible enzymes (e.g., heme oxygenase-1) (13), in response to electrophilic and reactive oxygen species-producing agents. However, growing evidence indicates that Nrf2 regulates a much broader gene expression response, including genes involved in protein homeostasis, such as chaperones and proteasome subunits (14–16), and thus, it is a potential target to deal with damaged proteins. It remains unknown if Nrf2 mediates a protective effect on different misfolded proteins by activating similar or divergent pathways.

Nrf2 mitigates neurodegeneration in genetic and toxin models of PD (17), and loss of Nrf2 exacerbates degeneration (12), implicating it as a potential therapeutic target of PD (18). Interestingly, the protective effect of Nrf2 is often mediated by expression of Nrf2 in astrocytes (19, 20), which supports a predominantly non–cell-autonomous mechanism of neuronal protection. Whether this protective effect by Nrf2 can be mediated directly in neurons has yet to be determined. Using a single-cell longitudinal imaging platform, we demonstrate that Nrf2 expression in neurons directly mitigates toxicity induced by α-synuclein and mutations in LRRK2 and that this effect is time-dependent. We visualized the metabolism and localization of α-synuclein and mutant LRRK2 at the level of a single cell. Nrf2 expression reduced α-synuclein levels by increasing the degradation of α-synuclein. In contrast, the reduction of mutant LRRK2 toxicity by Nrf2 was associated with an increased risk of IB formation and more insoluble and homogeneous IBs. The sequestering of LRRK2 into more compact IBs led to a drop in diffuse LRRK2 levels elsewhere in the cell. By shedding light on how Nrf2 mediates its protective effect on PD-associated protein toxicity, we also shed light on endogenous coping responses that might be bolstered to handle misfolded proteins.

Significance

The prevailing view of nuclear factor erythroid 2-related factor (Nrf2) function in the central nervous system is that it acts by a cell-nonautonomous mechanism to activate a program of gene expression that mitigates reactive oxygen species and the damage that ensues. Our work significantly expands the biological understanding of Nrf2 by showing that Nrf2 mitigates toxicity induced by α-synuclein and leucine-rich repeat kinase 2 (LRRK2), by potently promoting neuronal protein homeostasis in a cell-autonomous and time-dependent fashion. Nrf2 accelerates the clearance of α-synuclein, shortening its half-life and leading to lower overall levels of α-synuclein. By contrast, Nrf2 promotes the aggregation of LRRK2 into inclusion bodies, leading to a significant reduction in diffuse mutant LRRK2 levels elsewhere in the neuron.

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The authors declare no conflict of interest.

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See Commentary on page 803.

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Materials and Methods

Plasmids and Compounds. Information on the plasmids and compounds used are provided in *SI Materials and Methods*.

Cell Culture, Transfections, Immunocytochemistry, and Immunoblotting. Experimental details are provided in *SI Materials and Methods*.

Robotic Microscope Imaging System and Image Analysis. For neuronal survival analysis, images of neurons were taken at 12–24-h intervals after transfection with an automated imaging platform as described (21–23). Further information on image acquisition and image analysis is provided in *SI Materials and Methods*.

Statistical Analysis. With longitudinal single-cell data collected by tracking individual neurons over time, survival analysis was used to accurately determine differences in longevity between populations of cells (24). Further information on the statistical approaches used is provided in *SI Materials and Methods*.

Results

Nrf2 Overexpression Reduces LRRK2- and α-Synuclein–Induced Toxicity in Primary Rat Neurons. To elucidate a role for Nrf2 in cellular coping mechanisms for misfolded proteins, we developed a method to assay Nrf2 activation in live cells, including neurons. A common method to identify Nrf2 activation is through a reporter construct that contains the ARE (25) located in target genes of Nrf2. We adapted this approach by visualizing Nrf2 activation in real time in single cells by placing the expression of the fluorescent protein mApple under the control of the ARE sequence. To validate the ARE_mApple reporter gene, HEK293 cells were cotransfected with Venus, as a morphology marker, and ARE_mApple and treated with tertiary butylhydroquinone (tBHQ), an activator of endogenous Nrf2 (26). At 24 h posttransfection, cells were imaged, and ARE_mApple fluorescence was measured. Treatment with increasing doses of tBHQ caused a dose-dependent increase in ARE_mApple expression, indicative of ARE activation (Fig. S1*A*). Next we cotransfected HEK293 cells with Venus as a morphology marker, ARE_mApple, and either Nrf2 or empty vector and confirmed that overexpression of Nrf2 led to a robust activation of the ARE reporter in HEK293 cells (Fig. 1 *A* and *B*).

We then validated the ARE reporter in primary rat neurons, by first testing if we could activate endogenous Nrf2. Primary rat neurons were cotransfected with Venus and ARE_mApple and treated with tBHQ. At 24 h posttransfection, neurons showed a robust increase in ARE_mApple fluorescence (Fig. S1*B*). Next, we tested if Nrf2 overexpression could lead to ARE activation in neurons. As primary rat cultures contain some glia, Nrf2 activation in neurons might be caused by glia through a non–cell-autonomous mechanism. To determine the contribution of this mechanism, we labeled two cohorts of neurons in the same cultures with distinct fluorescent labels, pTag-BFP and GFP, via sequential transfections (Fig. 1*C*). The BFP-labeled cohort was also cotransfected with Nrf2, whereas GFP-labeled neurons were not. Both cohorts of neurons were also transfected with ARE_mApple to report Nrf2-dependent ARE activation. Similar to HEK293 cells, we found that neurons cotransfected with ARE_mApple, Nrf2, and pTag-BFP showed an up-regulation of ARE_mApple fluorescence (Fig. 1*D*). If glia were driving Nrf2-dependent ARE transcription in neurons, neurons ectopically expressing GFP but not Nrf2 should show an increase in ARE_mApple expression. However, we found that this cohort of neurons had a similar level of ARE activation as a population of neurons with no ectopic Nrf2 expressed in any of the cells. Interestingly, Nrf2 induced a 12.1-fold induction of ARE transcription in neurons, compared with only a 3.3-fold induction in HEK293 cells, suggesting potential differences in basal levels of Nrf2 in neurons and other cells types.

To test Nrf2's cell-autonomous role in neurons, we measured the expression of known Nrf2 targets, including those involved in stress response and protein homeostasis in neurons transfected with Nrf2. Primary rat cortical neurons were cotransfected with

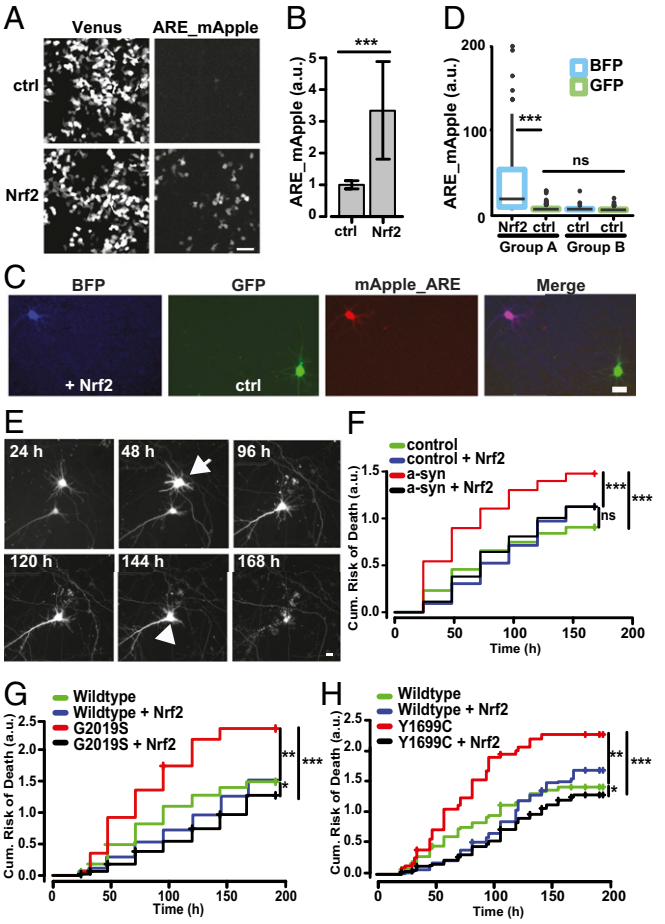

Fig. 1. Nrf2 activates ARE-dependent transcription in neurons and blocks degeneration caused by α-synuclein and mutant LRRK2. (*A*) HEK293 cells cotransfected with Venus and ARE-Apple. (Scale bar, 70 μm.) (*B*) Nrf2 overexpression up-regulated the ARE reporter, as measured by red fluorescence. Number of cells were as follows: control (ctrl) = 1,499; Nrf2 = 1,529. *F* value = 135.8, *df* = 1; ***$P <$ 0.0001; 95% confidence intervals (CIs). (*C*) Neurons were sequentially transfected with pTag_BFP and Nrf2 (BFP, +Nrf2), followed by GFP and control plasmid (GFP, +ctrl). Both cohorts of neurons were also cotransfected with ARE_mApple. (Scale bar, 10 μm.) (*D*) Only neurons overexpressing Nrf2 showed a significant up-regulation of ARE_mApple expression (group A, BFP Nrf2). Neurons with GFP and control plasmid (group A, GFP ctrl) had a similar level of ARE activation as a population of neurons with no ectopic Nrf2 expression (group B). Number of cells were as follows—group A, BFP Nrf2 = 58, GFP ctrl = 137; group B, BFP ctrl = 51, GFP ctrl = 102. *F* value = 19.66, *df* = 3; ***$P < 0e^{-10}$; ns, not significant; 95% CIs. (*E*) Images of two longitudinally tracked neurons expressing mRFP and α-synuclein. One neuron underwent neurite degeneration at 48–96 h (arrow); the other remains alive up until 144 h (arrow head). (Scale bar, 10 μm.) (*F*) Cumulative risk-of-death curves demonstrate that Nrf2 significantly reduced toxicity in neurons expressing α-synuclein. Number of neurons in α-synuclein = 640, α-synuclein + Nrf2 = 563, Control = 663, and Control + Nrf2 = 518. ***$P < 0e^{-10}$; ns, not significant. (*G* and *H*) Cumulative risk-of-death curves demonstrate that Nrf2 significantly reduced toxicity in neurons expressing mutant G2019S LRRK2 or Y1699C LRRK2. (*G*) Number of neurons for wild-type LRRK2 = 97, wild-type LRRK2 + Nrf2 = 165, G2019S LRRK2 = 171, and G2019S LRRK2 + Nrf2 = 319. *$P = 0.01$, **$P = 0.0004$, ***$P < 0e^{-10}$. (*H*) Number of neurons for wild-type LRRK2 = 366, wild-type LRRK2 + Nrf2 = 304, Y1699C LRRK2 = 139, and Y1699C LRRK2 + Nrf2 = 260. *$P = 5.42e^{-6}$, **$P = 0.00014$, ***$P < 0e^{-14}$.

mApple and Nrf2 or control plasmid and fixed 24 h later. Nrf2 overexpression caused a significant increase in expression of proteasome 20s alpha and GST (Fig. S2 *A–D*), known Nrf2 transcriptional targets (27). After validating Nrf2-dependent activation in neurons, we tested if overexpression of Nrf2 reduced toxicity induced by the PD-associated proteins LRRK2 and α-synuclein in

neurons. We examined Nrf2's effect on toxicity caused by two LRRK2 mutations: the most common PD-associated mutation, G2019S (28), and Y1699C. We showed that both mutations cause dose-dependent toxicity, neurite degeneration, and IB formation (5). To test Nrf2's effect on α-synuclein–induced toxicity, the wild-type α-synuclein protein was overexpressed. We found that overexpression of α-synuclein in neurons leads to dose-dependent toxicity (29), which models the duplication and triplication mutations of the α-synuclein gene that cause a severe, highly penetrant form of PD (30, 31).

α-Synuclein was cotransfected into primary rat neurons, along with Nrf2 and the morphology marker monomeric red fluorescent protein (mRFP). To ensure that the total DNA transfected into the neurons was the same across conditions, additional control plasmid was transfected into the cohort of neurons without exogenous Nrf2. Approximately 24 h after transfection, a robotic microscope (21) was used to collect consecutive images of the same fluorescent neurons. This approach enabled hundreds of neurons expressing mRFP, Nrf2, and α-synuclein to be longitudinally followed individually, which we did every day for 1 wk. The reproducible temporal expression profile of mRFP (Fig. S3) made it suitable to track neuron morphology (Fig. 1*E*). As shown in previous studies (21, 22), the loss of the mRFP signal coincided with the loss of membrane integrity (Fig. S4), indicating the time of death for each neuron. Similarly, we cotransfected primary rat neurons with Nrf2-, mRFP-, and either Venus-tagged mutant Y1669C, G2019S, or wild-type LRRK2 (Venus-LRRK2) and longitudinally imaged the cells once a day for 7 d and used the mRFP signal to determine the time of death of each neuron. Additional control plasmid was transfected into the cohort of neurons without exogenous Nrf2 to balance the DNA transfected into the neurons.

Using statistical survival tools that are commonly used in clinical studies (24), the time of death of each neuron within the different cohorts being followed was used to generate cumulative survival and risk-of-death curves. These plots revealed that neurons expressing α-synuclein had a greater cumulative risk-of-death and, thus, higher toxicity than control neurons (Fig. 1*F* and Fig. S5*A*) and that neurons overexpressing mutant LRRK2 had a greater risk of death than either control neurons or neurons expressing wild-type LRRK2 (Fig. 1 *G* and *H* and Fig. S5 *B* and *C*). Exogenous overexpression of Nrf2 caused a highly significant reduction in toxicity in neurons expressing either α-synuclein or mutant LRRK2 (Fig. 1 *F*–*H* and Fig. S5 *A*–*C*). Nrf2 also caused a small but statistically significant increase in survival of neurons overexpressing wild-type LRRK2 (Fig. 1 *G* and *H* and Fig. S5 *B* and *C*). However, Nrf2 does not seem to cause any dramatic improvement in baseline neuron viability, as Nrf2 did not improve the survival of control neurons (Fig. 1*F*). For Nrf2 to be considered as a potential therapeutic target, evidence that Nrf2 mitigates neurodegeneration in human neuron models is critical. Human induced pluripotent stem cells (iPSCs) derived from a healthy control individual were differentiated into human neurons (Fig. S5*D*). iPSC-derived human neurons were cotransfected with a panneuronal GFP_synapsin survival marker, α-synuclein, and either Nrf2 or control plasmid and longitudinally imaged for 7–9 d (Fig. S5*E*). Neurons expressing exogenous α-synuclein had a greater risk of death than control neurons. Nrf2 reduced the risk of death of neurons exogenously expressing α-synuclein without affecting the survival of control neurons (Fig. S5*F*). Our results highlight the specificity of Nrf2's protective effect on neurons undergoing neurodegeneration induced by PD-associated proteins. Furthermore, we show that Nrf2 can act directly in neurons and does not require a non–cell-autonomous mechanism of action.

The Protective Effect of Nrf2 Is Time-Dependent. Although we saw a strong protective effect of Nrf2 on PD-associated protein toxicity, we did observe that the cumulative risk-of-death curves of cells with or without Nrf2 expression diverged to a greater extent at the beginning of the experiment than at later times, hinting that Nrf2's effects might vary with time. We used Cox proportional hazard (CPH) analysis, a popular survival analysis method (32), to quantify both the magnitude of Nrf2's effect on toxicity and any adaptive changes in Nrf2's efficacy over time. The magnitude of Nrf2's effect on α-synuclein and mutant LRRK2-induced toxicity was surprisingly large (Table S1). Exogenous Nrf2 expression reduced the risk of death by 92% [hazard ratio (HR) of 0.082, $P = 5.6e^{-14}$] and 86% (HR of 0.142, $P = 1.2e^{-7}$) in neurons expressing G2019S and Y1699C LRRK2, respectively. Although, in neurons expressing α-synuclein, Nrf2 reduced the risk of death by 67% (HR = 0.33, $P < 2e^{-16}$). Furthermore, Nrf2's impact on PD-associated toxicity was most significant early on in the experiment and attenuated significantly with time (Table S1).

To determine if the time-dependent effect of Nrf2 was due to a decline in Nrf2 levels, we fixed cohorts of Nrf2 transfected neurons at 24 and 168 h posttransfection and measured levels of Nrf2 by immunocytochemistry. Nrf2 levels were comparable across both cohorts of neurons (Fig. S6*A*). Next, we investigated if the drop in efficacy could be due to a decrease in Nrf2-dependent ARE transcription. Neurons transfected with ARE_mApple were imaged and analyzed for ARE_mApple fluorescence at 24 and 168 h posttransfection. Interestingly, we found that levels of ARE_mApple were significantly lower at 168 h (Fig. S6*B*). To control for the confound that a decline in Nrf2-dependent ARE transcription could be due to the early death of neurons expressing high levels of ARE_mApple, neurons were cotransfected with ARE_mApple and GFP and longitudinally imaged for a week. The cotransfected GFP was used as a morphology marker to select only neurons that lived up to 172 h posttransfection (Fig. S6*C*). Neurons showed a significant decrease in ARE_mApple signal with time (Fig. S6 *C* and *D*), confirming a decrease in Nrf2-dependent ARE activation in neurons over time, despite a high level of Nrf2 expression. This suggests that neurons are undergoing an adaptive response to Nrf2 that eventually limits its signaling.

Nrf2 Increases α-Synuclein Turnover. As Nrf2 has been implicated in the up-regulation of targets involved in proteostasis, including molecular chaperones and protein degradation machinery (14, 15, 33–35), we next determined if Nrf2 mediated its strong protective effect on PD-associated protein toxicity in neurons by modulating α-synuclein and LRRK2 dynamics. With α-synuclein's propensity to misfold and induce a dose-dependent toxicity (29), Nrf2 may reduce α-synuclein toxicity by up-regulating protein degradation machinery to decrease α-synuclein levels (16). To measure the levels of diffuse α-synuclein in single cells, we tagged Dendra2 to α-synuclein and first confirmed that this fusion protein retained the dose-dependent toxicity (Fig. 2 *A*–*C*) that we previously showed for untagged α-synuclein (29). Although Dendra2–α-synuclein expression varied significantly from cell to cell, α-synuclein levels in single neurons measured by Dendra2_GFP fluorescence and by immunocytochemistry were highly correlated (Fig. S7 *A* and *B*), indicating that Dendra2 fluorescence provided a faithful estimate of the amount of protein in each cell. We also confirmed that Dendra2–α-synuclein was distributed throughout the cytosol, including expression in dendrites and spines of mature neurons (Fig. S7 *C* and *D*). We then transfected neurons with Dendra2–α-synuclein and Nrf2 or equal amounts of vector control. We found that cells expressing Nrf2 had lower steady-state levels of Dendra2–α-synuclein (Fig. 2*D*). We also measured Nrf2's effect on steady-state levels of untagged α-synuclein. Neurons cotransfected with untagged α-synuclein, mApple, and either Nrf2 or control were fixed at 24 h posttransfection and stained for α-synuclein. Similar to Dendra2–α-synuclein-transfected neurons, α-synuclein levels were lower in neurons transfected with Nrf2. In contrast, levels of cotransfected mApple fluorescence were similar (Fig. S7 *E*–*G*), indicating that Nrf2's effect was specific to α-synuclein and potentially indicating a role for Nrf2 in modulating α-synuclein turnover.

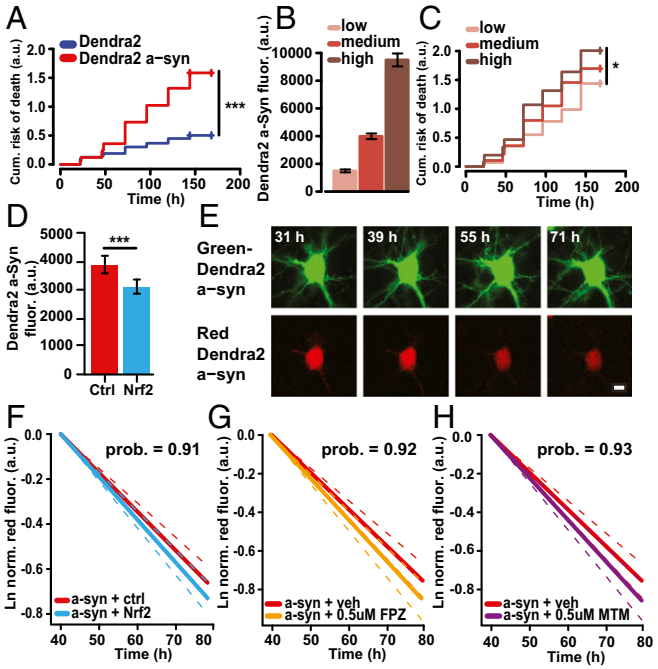

Fig. 2. Nrf2 decreases α-synuclein levels by increasing its degradation. (*A*) Overexpression of Dendra2–α-synuclein in neurons leads to an increased risk of death. Number of neurons: Dendra2 = 238, Dendra2–α-synuclein = 239; HR, 2.86; ***$P < 0.0001$. (*B*) Neurons expressing Dendra2–α-synuclein were separated into low-, medium-, or high-expressing neurons. (*C*) Neurons in the highest tercile of Dendra2–α-synuclein expression displayed higher risk of death than neurons in the lowest tercile of Dendra2–α-synuclein expression. Number of neurons in low = 59, medium = 60, and high = 67; HR, 1.54; low versus high *$P = 0.03$. (*D*) Steady-state levels of α-synuclein are lower in neurons expressing Nrf2. Number of neurons for ctrl = 139, Nrf2 = 96; *t* test, *F* value = 16.01; ***$P < 0.0001$. (*E*) A neuron transfected with Dendra2–α-synuclein was pulsed for 1–2 s with 405-nm light and imaged for several days. (Scale bar, 10 μm.) (*F*) Neurons expressing Nrf2 had a 10% increase in Dendra2–α-synuclein degradation. The mean slopes were –0.019 (95% CI –0.021, –0.017) and –0.0173 (95% CI, –0.019, –0.015) for Nrf2 and control cells, respectively. The posterior probability for a steeper mean slope of RFP fluorescence in Nrf2 cells was 0.91, indicating that it is overwhelmingly likely that the degradation of Dendra2–α-synuclein was faster in cells with Nrf2. Number of neurons, Dendra2–α-synuclein/Ctrl = 400 and Dendra2–α-synuclein/Nrf2 = 377. (*G* and *H*) Neurons transfected with Dendra2–α-synuclein and exposed to 0.5 μM fluphenazine (FPZ) or 0.5 μM methotrimeprazine (MTM) show a 24% (posterior probability, 0.92) and 23% (posterior probability, 0.93) faster degradation of α-synuclein, respectively, compared with vehicle control (DMSO). The mean slopes for RFP fluorescence were –0.0192 (CI, –0.021, –0.017), –0.0216 (CI, –0.024 to 0.019), and –0.0218 (CI, –0.024 to 0.019) for neurons treated with vehicle (veh), 0.5 μM FPZ, and 0.5 μM MTM, respectively. Number of neurons in veh = 490, FPZ = 337, and MTM = 340.

Steady-state levels of α-synuclein result from a balance of production and degradation. As our synuclein expression constructs are not expected to be activated by Nrf2 because they evidently lack AREs, we hypothesized that Nrf2 lowers steady-state synuclein levels by accelerating its degradation. We measured α-synuclein degradation rates in individual neurons by optical pulse labeling (16, 36). This approach takes advantage of the photoswitchable properties of Dendra2 in which an irreversible change of its spectral properties occurs after illumination with short-wavelength light. In our model, cells expressing Dendra2–α-synuclein initially fluoresce green. Upon photoswitching, a population of the green fluorescent protein is irreversibly switched to red, creating a distinct intracellular population of photoconverted α-synuclein that can be followed by the automated longitudinal analysis (Fig. 2*E* and Fig. S8*A*). Red fluorescence of Dendra2–α-synuclein was measured longitudinally within individual cells. Using a Bayesian hierarchical model, measurements from hundreds of cells were used to model the underlying degradation of α-synuclein in the population, enabling us to determine if the presence of Nrf2 modulated α-synuclein degradation in single cells. In cells overexpressing Nrf2, the degradation of α-synuclein was 10% faster (posterior probability, 0.92) (Fig. 2*F* and Fig. S8*B*). As a comparison, we exposed primary rat neurons expressing α-synuclein to a novel family of small-molecule autophagy inducers, namely FPZ and MTM, which we previously showed to up-regulate autophagy in primary rat cortical neurons (36). FPZ and MTM led to a 24% (posterior probability, 0.92) and 23% (posterior probability, 0.93) faster degradation of α-synuclein, respectively (Fig. 2 *G* and *H* and Fig. S8 *C* and *D*). We next asked if the benefits of Nrf2 might be explained through its effects on α-synuclein levels. Using CPH analysis, we compared the beneficial effect of Nrf2 on α-synuclein toxicity before and after adjustment for α-synuclein levels. Before incorporating α-synuclein levels into the model, Nrf2 significantly reduces α-synuclein–induced toxicity; however, once α-synuclein levels are incorporated into the model, this significance drops away (Table S2), indicating the Nrf2's beneficial effect on α-synuclein toxicity can be explained by its effects on α-synuclein levels.

Nrf2 Increases LRRK2 IB Formation and Insolubility. Next, we determined if Nrf2-mediated protection leads to a similar effect on LRRK2 dynamics. We began by looking at steady-state levels of LRRK2, by measuring Venus-LRRK2 levels in neurons cotransfected with mutant Venus-LRRK2 and either Nrf2 or control. In contrast to α-synuclein, Nrf2 did not lead to a detectable decrease in steady-state levels of diffuse mutant LRRK2 at 24 h posttransfection (Fig. 3*A*). In addition to targeting misfolded proteins for degradation, toxic protein conformers may be sequestered into IBs enabling nonnative toxic epitopes to be buried or refolded (21, 37). Similar to other disease-causing misfolded proteins, LRRK2 has a propensity to aggregate and form IBs (38, 39). Thus, we wanted to determine if the protective effect of Nrf2 is mediated through the shunting of diffuse mutant LRRK2 into IBs, thereby increasing LRRK2 IB formation. To test this, we used our longitudinal imaging platform to track Venus-LRRK2 IB formation in real time. For each neuron expressing mutant Venus-LRRK2, we recorded if and when an IB formed (Fig. S9). As death can preclude IB formation, we plotted the cumulative risk of IB formation, using a semiparametric proportional hazards model that incorporates competing effects, such as death. When we compared the risk of IB formation in neurons expressing mutant Venus-G2019S-LRRK2 or Venus-Y1699C-LRRK2 with and without Nrf2, we found that Nrf2 led to a significantly greater risk of IB formation for the PD-associated LRRK2 mutants (Fig. 3*B*).

The targeting of IBs to specific cellular locations is considered an additional line of defense in the handling of misfolded proteins (40, 41). In the absence of Nrf2, LRRK2 IBs were more heterogeneous and dispersed throughout the cytoplasm, and the morphology differed from cell to cell (Fig. 3*C*). However, in the presence of Nrf2, LRRK2 IBs were predominantly restricted to near the nucleus as compact foci (Fig. 3*C*). The intracellular location of protein IBs is a known predictor of IB insolubility (40). Interestingly, insolubility in high concentrations of detergent is an important characteristic of many protein IBs found in neurodegenerative diseases (42), and in the case of prion disease, insolubility is correlated with slower disease progression (43). Thus, to evaluate the insolubility of LRRK2 IBs, we treated neurons with high concentrations of detergent (2.5% SDS/2.5% Triton-X-100) (44), which revealed that in the presence of Nrf2, LRRK2 IBs were more insoluble than those that formed without Nrf2 (Fig. 3*D*).

We previously showed that diffuse LRRK2 is toxic in a dose-dependent manner (5), so we hypothesized that Nrf2 may reduce mutant LRRK2-associated toxicity by sequestering misfolded

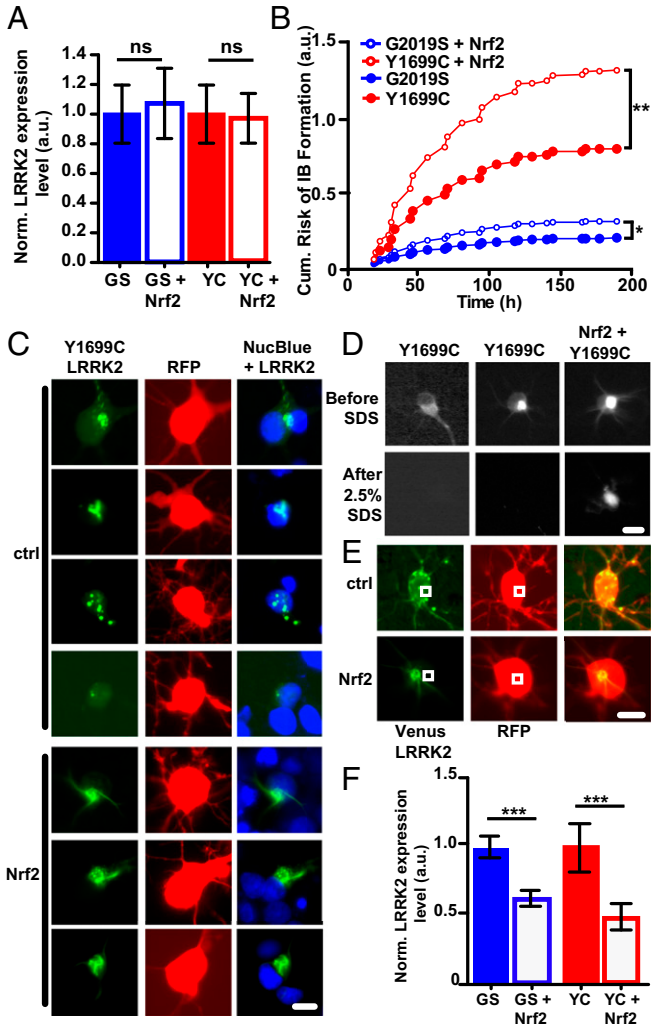

Fig. 3. Nrf2 increases LRRK2 IB formation and insolubility. (*A*) At 24 h posttransfection, levels of G2019S-LRRK2 and Y1699C-LRRK2 were unchanged in the presence of Nrf2. Number of neurons per group for G2019S (GS) = 200, GS + Nrf2 = 316, Y1699C (YC) = 128, and YC + Nrf2 = 247; 95% CIs. ns, not significant. (*B*) Nrf2 increases the cumulative risk of IB formation in neurons expressing G2019S-LRRK2 and Y1699C-LRRK2. $*P < 0.01$, $**P < 0.001$. (*C*) Representative images of neurons expressing Y1699C-LRRK2, mRFP, and either Nrf2 or empty vector control at 48–72 h posttransfection. Neurons were treated with NucBlue to label the nuclei. Neurons expressing Nrf2 form more homogeneous, perinuclear, and compact IBs. (Scale bar, 10 μm.) (*D*) A detergent resistance assay shows that, in the presence of Nrf2, G2019S-LRRK2 IBs are resistant to SDS/Triton X-100 treatment. (Scale bar, 10 μm.) (*E*) Representative images of neurons cotransfected with Y1699C-LRRK2, RFP, and either Nrf2 or control plasmid at 32–45 h posttransfection. Neurons with IBs were measured for diffuse levels of Venus-LRRK2 elsewhere in the cell (white boxes). (Scale bar, 10 μm.) (*F*) Neurons that form IBs by 32 h posttransfection in the presence of Nrf2 have significantly lower levels of cytoplasmic diffuse G2019S-LRRK2 and Y1699C-LRRK2 than neurons with no Nrf2. Number of cells per group were GS = 26, GS + Nrf2 = 27, YC = 52, and YC + Nrf2 = 68; 95% CIs; *t* test; *F* values 14.93 (G2019S) and 18.08 (Y1699C). $***P = 0.00038$ (G2019S) and $P = 4.46e^{-0.5}$ (Y1699C).

diffuse LRRK2 into more compact IBs. To test this, we measured levels of diffuse LRRK2 in neurons that had formed IBs by 32 h posttransfection (Fig. 3*E*). We found that, in the presence of Nrf2, neurons with IBs had a significant reduction in diffuse mutant LRRK2 levels elsewhere in the cell (Fig. 3*F*). These findings indicate that a reduction in PD-associated LRRK2 toxicity by Nrf2 is associated with an augmented risk of IB formation, a reduction in the heterogeneity of IBs, and an increase in IB detergent resistance and improved survival. This suggests that Nrf2 promotes a shift of LRRK2 from the diffuse fraction into IBs and a shift from less ordered IBs to more ordered ones.

Discussion

The prevailing view of Nrf2's function in the central nervous system is that it acts by a cell-nonautonomous mechanism to activate genes that mitigate reactive oxygen species. However, growing evidence indicates that Nrf2 can act in neurons via a cell-autonomous manner, leading to transcriptional changes in not only oxidative stress-related genes but also those related to proteostasis and specific neuronal functions (45–47). Our work demonstrates that Nrf2 can act in a cell-autonomous manner to potently promote neuronal protein homeostasis and reduce neurodegeneration caused by α-synuclein and mutant LRRK2 expression.

Our study provides supporting evidence for Nrf2's role in the progression and risk of PD. Genetic association studies linked single nucleotide polymorphisms that lower Nrf2 promoter activity (48) with an increased risk of PD (49). Nrf2 was up-regulated in neurons differentiated from iPSCs derived from PD patients with mutations in Parkin, another PD-associated gene (50). Furthermore, accumulation of Nrf2 and its targets has been observed in surviving dopaminergic neurons in the substantia nigra of PD patients (51, 52), indicating that the Nrf2 pathway is activated in neurons during the neurodegenerative process. Previous studies in PD models focused on Nrf2's non–cell-autonomous protective effect on neurons, where expression of Nrf2 in astrocytes reduced neuron death caused by toxins (20) or mutant A53T α-synuclein (53). In contrast to previous studies, we altered expression of Nrf2 directly in primary rat neurons and showed that increased expression of Nrf2 and subsequent activation of ARE transcription was sufficient to activate endogenous stress response mechanisms, leading to improved survival of neurons against toxicity caused by both mutant LRRK2 and α-synuclein.

We also address the effects of chronic activation of Nrf2. Nrf2's effect on α-synuclein– and LRRK2-induced degeneration attenuated with time and was associated with a time-dependent decrease in Nrf2-dependent transcription, despite a steady level of Nrf2 expression. This suggests that neurons are undergoing an adaptive response to Nrf2 that eventually limits its signaling. Although Nrf2 displays time-dependent effects on PD-associated phenotypes, it does not eliminate the possibility that Nrf2 may be used as a therapeutic strategy in a pulsatile or periodic manner to maximize its benefits and avoid detrimental effects.

Nrf2's protective effect on neurodegeneration was accompanied by changes in the dynamics of LRRK2 and α-synuclein, strengthening the concept that activation of endogenous defense mechanisms protect against proteostasis stress (54) and could be harnessed for therapeutic intervention. In addition, we support growing evidence that, in addition to antioxidant genes (55), Nrf2 up-regulates genes involved in proteostasis, including components of the proteasome (56, 57) and chaperone systems (15, 27). Interestingly Nrf2's effect on protein dynamics differed for α-synuclein and LRRK2. Nrf2 accelerates the clearance of α-synuclein, shortening its half-life and leading to lower overall levels of α-synuclein. By contrast, Nrf2 promotes the aggregation of LRRK2 into IBs and promotes the formation of IBs that are more compact and insoluble. How Nrf2 leads to different prominent effects on α-synuclein and LRRK2 dynamics remains unclear. As Nrf2 is activated by a battery of cellular stresses, including perturbations to proteostasis (35), the distinct species of misfolded protein might influence the distinct cellular responses activated by Nrf2. Alternatively Nrf2 may activate the same broad program of gene expression, which includes multiple aspects of protein homeostasis, for both α-synuclein and LRRK2. The differences we observe in terms of Nrf2's effects on α-synuclein versus LRRK2 might relate to how those two proteins interact with different components of the Nrf2 program. Further investigation of the downstream targets that are activated

by Nrf2 during stress-specific degeneration in neurons will provide greater insight into pathways or networks that can be modulated as a therapeutic strategy against PD-associated proteotoxicity.

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