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Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson's disease

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14-3-3 proteins are important negative regulators of cell death pathways. Recent studies have revealed alterations in 14-3-3s in Parkinson's disease (PD) and the ability of 14-3-3s to interact with α-synuclein (α-syn), a protein central to PD pathophysiology. In a transgenic α-syn mouse model, we found reduced expression of 14-3-3θ, -ε, and -γ. These same isoforms prevent α-syn inclusion formation in an H4 neuroglioma cell model. Using dopaminergic cell lines stably overexpressing each 14-3-3 isoform, we found that overexpression of 14-3-3θ, -ε, or -γ led to resistance to both rotenone and 1-methyl-4-phenylpyridinium, whereas other isoforms were not protective against both toxins. Inhibition of a single protective isoform, 14-3-3θ, by shRNA did not increase vulnerability to neurotoxic injury, but toxicity was enhanced by broad-based inhibition of 14-3-3 action with the peptide inhibitor difopein. Using a transgenic *C. elegans* model of PD, we confirmed the ability of both human 14-3-3θ and a *C. elegans* 14-3-3 homologue (*ftt-2*) to protect dopaminergic neurons from α-syn toxicity. Collectively, these data show a strong neuroprotective effect of enhanced 14-3-3 expression – particularly of the 14-3-3θ, -ε, and -γ isoforms – in multiple cellular and animal models of PD, and point to the potential value of these proteins in the development of neuroprotective therapies for human PD.
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Parkinson's disease (PD) is a neurodegenerative disorder affecting more than four million older individuals in the most populous nations.[1] There is currently no treatment that slows the degenerative process. Although the cause of most PD cases is unknown, substantial evidence points to a central role of the protein α-synuclein (α-syn). Families with point mutations or gene multiplication of α-syn exhibit autosomal dominant PD.[2–6] In sporadic PD, α-syn aggregates are present in Lewy bodies.[7,8] α-Syn overexpression in cellular and animal models leads to cellular injury and death,[9–12] but the mechanisms of this toxicity are poorly understood.

In an effort to identify factors contributing to α-syn toxicity, we have previously evaluated alterations in gene expression in the substantia nigra (SN) of transgenic mice overexpressing human wild-type α-syn.[13] We observed that among altered genes are those encoding the 14-3-3 proteins, a family of highly conserved proteins. There are seven mammalian 14-3-3 isoforms, comprising 1% of total brain protein, and these proteins participate in many cellular functions by mediating protein–protein interactions.[14,15] 14-3-3s has a central role in cell survival, and 14-3-3 depletion can lead to activation of proapoptotic factors.[16]

The observation that 14-3-3 expression was dysregulated in a rodent model of α-syn overexpression was of particular interest because of other evidence linking 14-3-3s to PD. 14-3-3s share structural homology with α-syn,[17] and co-aggregate with α-syn in Lewy bodies in human PD[18] and in a mouse PD model.[19] 14-3-3 and α-syn can be co-immunoprecipitated from mammalian brains,[11,17] and co-immunoprecipitation is increased in PD brains.[20] 14-3-3η is a negative regulator of the protein parkin,[20] mutation of which leads to early onset PD.[21]

Together, these observations support the hypothesis that α-syn toxicity can arise from sequestration of 14-3-3 proteins, disrupting cellular signaling, and liberating proapoptotic factors. To test this hypothesis, we have examined the expression of each 14-3-3 isoform in α-syn transgenic mice and evaluated the effects of selective expression of the different isoforms in PD cellular models and in an invertebrate system. Our data reveal a potent and selective inhibition of toxicity by a subset of the 14-3-3 isoforms: 14-3-3θ, -ε, and -γ.

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Results

14-3-3 isoform expression in α-syn transgenic mice. We had previously examined changes in overall gene expression in the SN of α-syn transgenic mice and found that distinct 14-3-3 isoforms were among the several hundred genes whose expression was altered in this gene array study.[13] As this mouse model yields most prominent α-syn pathology in cortical neurons, we assessed the expression patterns of 14-3-3 isoforms in cortical homogenates from 3-month-old transgenic mice, using quantitative PCR. We found that 14-3-3θ was the most significantly downregulated isoform in the cortex of the transgenic mice. This isoform was decreased by nearly 50% in transgenic mice compared to wild-type mice (Figure 1; $P<0.05$). 14-3-3γ was reduced to 60% of wild type ($P<0.05$). Expression of 14-3-3ε and 14-3-3σ trended downward but did not reach statistical significance ($P=0.072$ for 14-3-3ε; $P=0.088$ for 14-3-3σ).

14-3-3ε, -γ, and -θ reduce α-syn inclusion formation. Because previous studies have revealed that α-syn and 14-3-3s are colocalized in Lewy bodies, we asked whether increased 14-3-3 expression could affect α-syn aggregation. We used an *in vitro* α-syn inclusion body assay developed by McLean *et al.*[22] Transfection of α-syn/truncated green fluorescent protein fusion (synT) with synphilin results in cytoplasmic inclusions that immunostain for α-syn in about 50% of H4 neuroglioma cells (Figure 2). We co-transfected H4 cells with either empty vector or a 14-3-3 isoform along with synT and synphilin and immunostained cells against α-syn 24 h after transfection. 14-3-3ε, -γ, and -θ reduced the number of cells with α-syn inclusions by 40% compared to control (Figure 2e; $P<0.001$). The other isoforms did not affect α-syn inclusion formation. The reduction in inclusion formation was not secondary to reduced α-syn expression, as expression levels of synT were comparable between control and 14-3-3θ-transfected cells (Figure 2f). Levels

of endogenous monomeric α-syn were also not reduced in 14-3-3θ-transfected cells (data not shown).

Creation of 14-3-3 stable cell lines. To examine directly the potential neuroprotective properties of 14-3-3s, we created stable cell lines overexpressing each 14-3-3 isoform. We subcloned cDNA clones for the 14-3-3 isoforms into the expression vector pcDNA3.1/V5-His. The 14-3-3 isoforms expressed from this vector are tagged with V5 and His$_6$ epitopes at the C-terminal end. SK-N-BE(2)-M17 (M17) dopaminergic cells were transfected with each 14-3-3 vector, and stable clones were selected in the presence of G418. We created approximately 15–20 clones for each isoform. Expression of the 14-3-3 proteins was assessed by western blot analysis (Figure 3), and we chose 2–3 high-expressing clones for the experiments described below. We also created a control stable line by transfecting M17 cells with the empty pcDNA3.1/V5-His plasmid.

14-3-3 overexpression in these stably transfected lines did not affect the subcellular distribution of 14-3-3s. Immuno-staining of naive M17 cells (Figure 4a) or control stable cells (Figure 4b) with an antibody against 14-3-3θ revealed a predominantly cytoplasmic distribution. A similar distribution was seen when we immunostained 14-3-3θ-overexpressing stable cells with either the 14-3-3θ antibody (Figure 4c) or a V5 antibody (Figure 4f). Staining against 14-3-3ε revealed similar subcellular distribution of 14-3-3ε in naive M17 and 14-3-3ε-overexpressing stable cells (data not shown). Subcellular distribution of α-syn was also not altered in 14-3-3θ stable cells as compared to control cells (Figure 4g–j).

14-3-3θ overexpression reduces rotenone toxicity. Rotenone is a pesticide that induces a parkinsonian syndrome in animals and reliably produces dose-dependent α-syn aggregation and injury.[23,24] Because 14-3-3θ was the isoform most altered in the cortex of α-syn transgenic mice, we first assessed the effect of 14-3-3θ on vulnerability to rotenone. Control and 14-3-3θ-overexpressing cells were incubated with varying concentrations of rotenone in serum-free DMEM for 24, 30, and 48 h. Cell death was assayed by lactate dehydrogenase (LDH) release into the culture media. We found that rotenone produced dose-dependent toxicity, and that 14-3-3θ cells were more resistant to rotenone than were control cells over a range of rotenone doses and time points (Figure 5). The difference between control and 14-3-3θ cells was most prominent at 48 h, when cell death in response to 1 μM rotenone in the 14-3-3θ cells was reduced to 45% of that in control cells (Figure 5c). We confirmed these findings with a second 14-3-3θ clone (Figure 5d).

As a control for artifacts that may have been introduced during the selection of stable cell lines, we also examined the effects of transient 14-3-3θ transfection into naive M17 cells, using transfection with EYFP for comparison. Because this method leads to low rates of transfection, we used a different approach to measure cell death. Cells were stained against V5 or green fluorescent protein (GFP) after the treatment period, and cell injury was assessed by Hoechst 33342 staining (Invitrogen, Carlsbad, CA, USA). With the rater masked to experimental condition, the nuclei of cells that

Figure 1 14-3-3 Expression in transgenic α-syn mice and wild-type littermates. 14-3-3θ and γ mRNA levels were significantly reduced in α-syn transgenic mice compared with wild-type mice. RNA was extracted from the cortex of 3-month-old transgenic mice and wild-type littermates. Primers specific to each 14-3-3 isoform were used for quantitative PCR to determine the amount of each 14-3-3 transcript. Results were normalized to GAPDH. $n=8$ per group. *$P<0.05$ (least-squares means test). Error bar reflects S.E.M.

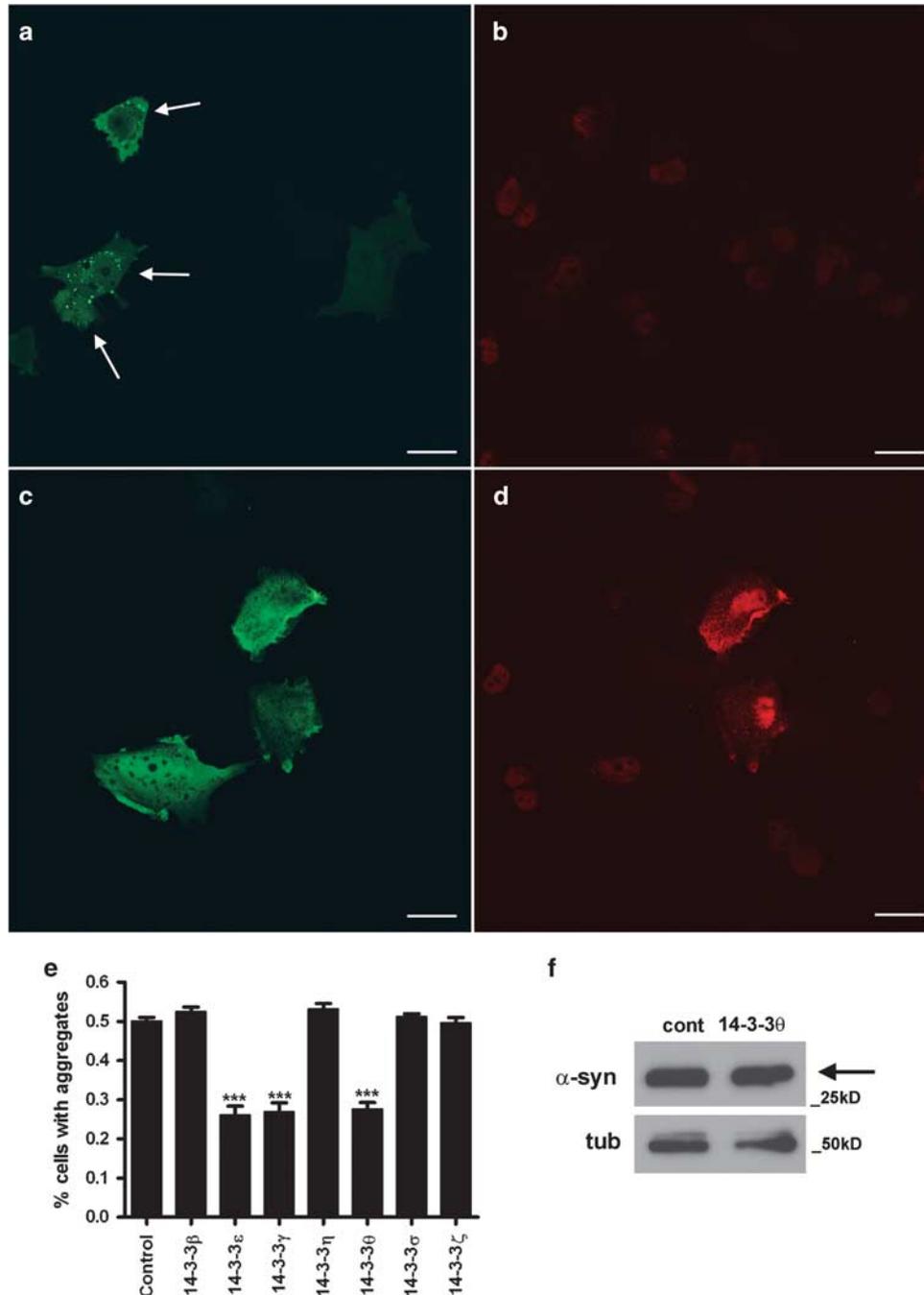**Figure 2** 14-3-3ε, -γ, and -θ reduce α-syn inclusions in H4 neuroglioma cells. (**a–d**) Immunocytochemistry of H4 cells transfected with synT, synphilin, and empty vector (**a**, **b**) or 14-3-3θ (**c**, **d**) with an antibody against α-syn and an Alexa 488-conjugated goat anti-mouse secondary antibody (green; **a**, **c**) or a pan-14-3-3 antibody and a cy-3-conjugated goat anti-rabbit secondary antibody (red; **b**, **d**). Fewer cells transfected with 14-3-3θ show α-syn inclusions as compared to control cells. Arrows point to cells with α-syn inclusions. Scale bar = 25 μM. (**e**) H4 cells were co-transfected with either empty vector or a 14-3-3 isoform along with synT and synphilin. At 24 h after transfection, cells were fixed and stained with a monoclonal antibody against α-syn. Cells that stained for α-syn were scored as positive or negative for α-syn inclusions, with the rater masked to experimental condition. Results reflect three independent experiments with four replicates per experiment. ***$P < 0.001$ (Bonferroni's multiple comparison test). Error bars reflect S.E.M. (**f**) Expression levels of synT are not affected in H4 cells transfected with 14-3-3θ. Representative western blot of total lysates from empty vector and 14-3-3θ transfected H4 cells incubated with an α-syn antibody is shown. Results were confirmed in three independent experiments. Arrow points to the $\sim$32 kDa α-syn/GFP fusion protein (synT). Tubulin was used as a loading control

stained for V5 or GFP were scored as normal or apoptotic. Cells transiently transfected with 14-3-3θ-overexpressing cells showed decreased cell death in response to rotenone (Figure 5e).

Given our results in the α-syn inclusion assay, we next evaluated whether levels of insoluble α-syn were reduced in 14-3-3θ cells as compared to control cells in response to rotenone. Rotenone increases insoluble α-syn both *in vitro*

a

14-3-3θ clones

b

c
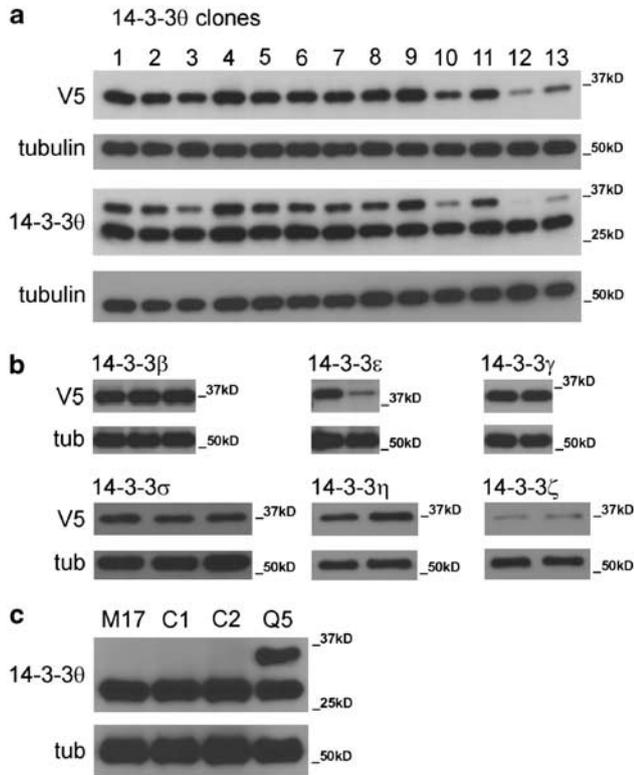
Figure 3 Stable M17 cells created to overexpress each 14-3-3 isoform. (**a**) 14-3-3θ was subcloned into the pcDNA3.1/V5-His vector, and SK-N-BE(2)-M17 cells were transfected with V5/His-tagged 14-3-3θ construct. Cells stably transfected with 14-3-3θ were selected in the presence of G418. A total of 13 different clones were analyzed for their expression of 14-3-3θ. Protein lysates of these clones were blotted with a monoclonal antibody against V5 to detect exogenous 14-3-3θ (top blot) or with a monoclonal antibody against 14-3-3θ to detect total 14-3-3θ levels (exogenous or endogenous; bottom blot). Two clones (clones 4 and 5) with high levels of 14-3-3θ overexpression were used for the LDH experiments described below. (**b**) 15–20 stable clones for each of the other 14-3-3 isoforms were similarly created and analyzed for 14-3-3 overexpression by western blot against the V5 epitope tag. Two to three clones for each 14-3-3 isoform were selected for the LDH experiments based on high expression levels. Western blots of these selected clones are shown using an antibody against V5. (**c**) Immunoblotting against 14-3-3θ reveals higher expression level of 14-3-3θ in 14-3-3θ stable cell line clone 5 (Q5) as compared to untransfected naive M17 cells (M17) and two control stable lines (C1, C2). Tubulin was used as a loading control for all blots

and *in vivo*.[23,24] We treated control and 14-3-3θ cells with 5 μM rotenone for 24 h and then separated cell lysates into Triton X-100 soluble and insoluble fractions. Upon rotenone treatment, insoluble α-syn was dramatically increased in control cells, whereas a much smaller increase in insoluble α-syn was seen in 14-3-3θ cells treated with rotenone (Figure 5f).

We next examined whether 14-3-3θ reduction by lentiviral shRNAs would result in increased rotenone toxicity to M17 cells. 14-3-3θ shRNA lentiviruses significantly reduced 14-3-3θ in M17 cells as compared to control virus with the empty pLKO.1 vector (Supplementary Figure 1a). Cells infected with 14-3-3θ shRNA did not show increased rotenone toxicity compared to control cells (Supplementary Figure 1b). These results were confirmed using another 14-3-3θ shRNA in the same lentiviral construct (data not shown).

Differential effects of overexpression of 14-3-3 isoforms on rotenone toxicity. Given the lack of increased rotenone toxicity with 14-3-3θ knockdown, we hypothesized that other 14-3-3 isoforms could compensate for the loss of 14-3-3θ. We decided to test whether overexpression of other 14-3-3 isoforms could protect against rotenone toxicity. We found significant differences between the different isoforms in terms of their ability to protect against rotenone. Cells over-expressing 14-3-3ε and -γ showed prominent reduction in rotenone-induced toxicity (Figure 6a and b). Rotenone-induced cell death at 1 μM was reduced to 65 and 63% of control in the 14-3-3ε and 14-3-3γ lines, respectively. Overexpression of 14-3-3β and -ζ also provided some protection, but not to the same extent as 14-3-3ε, -γ, or -θ (Figure 6c and d). Rotenone-induced cell death at 1 μM was reduced to 70 and 78% of control levels in 14-3-3β and 14-3-3ζ cells, respectively. Differences in protection did not correlate with 14-3-3 expression levels, as 14-3-3β cells had higher V5 staining on western blots compared to 14-3-3ε and -θ (Figure 3). Results for each isoform were confirmed in 1–2 other cell clones overexpressing that isoform (data not shown).

Overexpression of 14-3-3η or -σ did not provide protection against rotenone at any doses tested (Figure 6e and f). Instead, 14-3-3η and 14-3-3σ cells showed increased cell death when maintained in serum-free media ($P < 0.01$ and < 0.001, respectively) compared with control. In the presence of rotenone, 14-3-3σ cells showed increased toxicity compared to control cells, although the difference between 14-3-3σ and control cells was less prominent. The 14-3-3η cells showed a nonsignificant trend toward increased toxicity in the presence of rotenone compared to control cells. These findings suggest that the 14-3-3η and -σ cells were more sensitive to serum deprivation *per se* and that the over-expression of these proteins does not confer any specific resistance to rotenone. The results for 14-3-3η and -σ were confirmed in 1–2 other cell clones overexpressing that isoform (data not shown).

Difopein promotes rotenone toxicity. As several 14-3-3 isoforms are protective against rotenone, we next tested whether a global competitive inhibitor of 14-3-3 function would promote rotenone toxicity. Difopein, or dimeric, fourteen-three-three peptide inhibitor, is a high-affinity 14-3-3 antagonist that disrupts 14-3-3/ligand interactions.[25] We subcloned the difopein-EYFP sequence into the tetracycline-inducible lentiviral construct pSLIK[26] to allow regulated induction of difopein, as it causes apoptosis at baseline.[25] As the control, we subcloned the scrambled difopein sequence with EYFP into pSLIK. M17 cells were infected with either the difopein-EYFP lentivirus or the control-EYFP lentivirus, and infected cells were selected in the presence of hygromycin. Two days before treatment with rotenone, selected cells were treated with 10 μg/ml doxycycline to induce difopein (or scrambled difopein) expression, and then cells were incubated with rotenone for 30 h. We found that cells expressing difopein showed increased sensitivity to rotenone compared to control cells (Figure 6g).
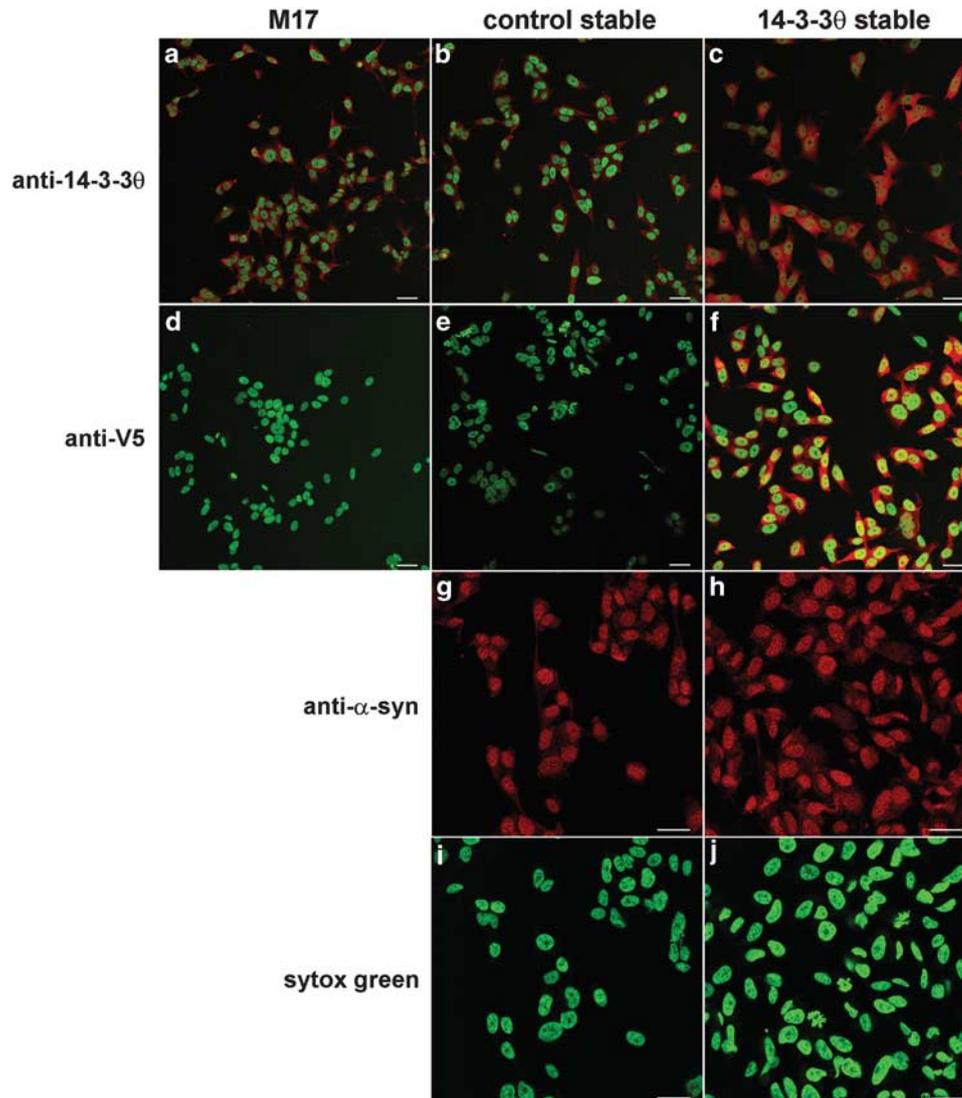
Figure 4 Subcellular distribution of V5-tagged 14-3-3 isoforms and α-syn in stable cell lines was similar to endogenous expression in control M17 cells. (**a–c**) Naive M17 cells (**a**), control stable cells (**b**), and 14-3-3θ-overexpressing cells (**c**) were immunostained using a primary monoclonal antibody against 14-3-3θ and a cy-3-conjugated goat anti-mouse secondary antibody to determine the cellular distribution of endogenous and overexpressed 14-3-3θ. Cells were also stained with Sytox Green to localize nuclei. 14-3-3θ Expression in all cells was seen predominantly in the cytoplasm. (**d–f**) These cell lines were also stained with a primary monoclonal antibody against V5 and a cy3-conjugated goat anti-mouse secondary antibody to determine the cellular distribution of exogenous V5-tagged 14-3-3θ. V5 staining showed similar cellular distribution as 14-3-3θ in 14-3-3θ stable cells (**f**). No V5 staining was apparent in M17 (**d**) or control stable cells (**e**). Cells were stained with Sytox Green to visualize nuclei. (**g–j**) Control cells (**g**, **i**) and 14-3-3θ stable cells (**h**, **j**) were stained with an antibody against α-syn to determine subcellular localization of α-syn. Staining was apparent in both nuclear and cytoplasmic regions for both control (**g**) and 14-3-3θ (**h**) cells. Sytox Green was used to stain nuclei (**i**, **j**). Scale bar = 30 μM

Differential effects of overexpression of 14-3-3 isoforms on MPP⁺ toxicity. We next tested whether the protective effects of certain 14-3-3 isoforms were restricted to rotenone or also effective against other neurotoxins that cause parkinsonian syndromes in animals. 1-Methyl-4-phenyl-pyridinium (MPP⁺) is the active metabolite of MPTP and causes dose-dependent cell death in dopaminergic cell lines. We tested the effects of MPP⁺ on all seven cell lines. Once again, 14-3-3θ, -ε, and -γ overexpression reduced toxicity in response to MPP⁺ treatment for 24 h (Figure 7a–c). 14-3-3β and -ζ overexpression did not show any significant protection against MPP⁺ (Figure 7d and e). 14-3-3η and -σ cells also showed similar levels of cell death compared with control

cells in response to MPP⁺ (Figure 7f and g). Once again, these two sets of lines showed increased toxicity compared to control cells in serum-free media in the absence of MPP⁺.

Effects of 14-3-3s on α-syn toxicity *in vivo*. Although both rotenone and MPP⁺ induce α-syn aggregation, we wished to assess more directly the potential protective effects of 14-3-3s against α-syn toxicity. We turned to a *C. elegans* model of PD, in which human wild-type α-syn and GFP are overexpressed in dopaminergic neurons under the control of the dopamine transporter (*dat-1*) promoter. Transgenic *C. elegans* overexpressing human wild-type α-syn show a predictable loss of dopaminergic neurons as indicated by
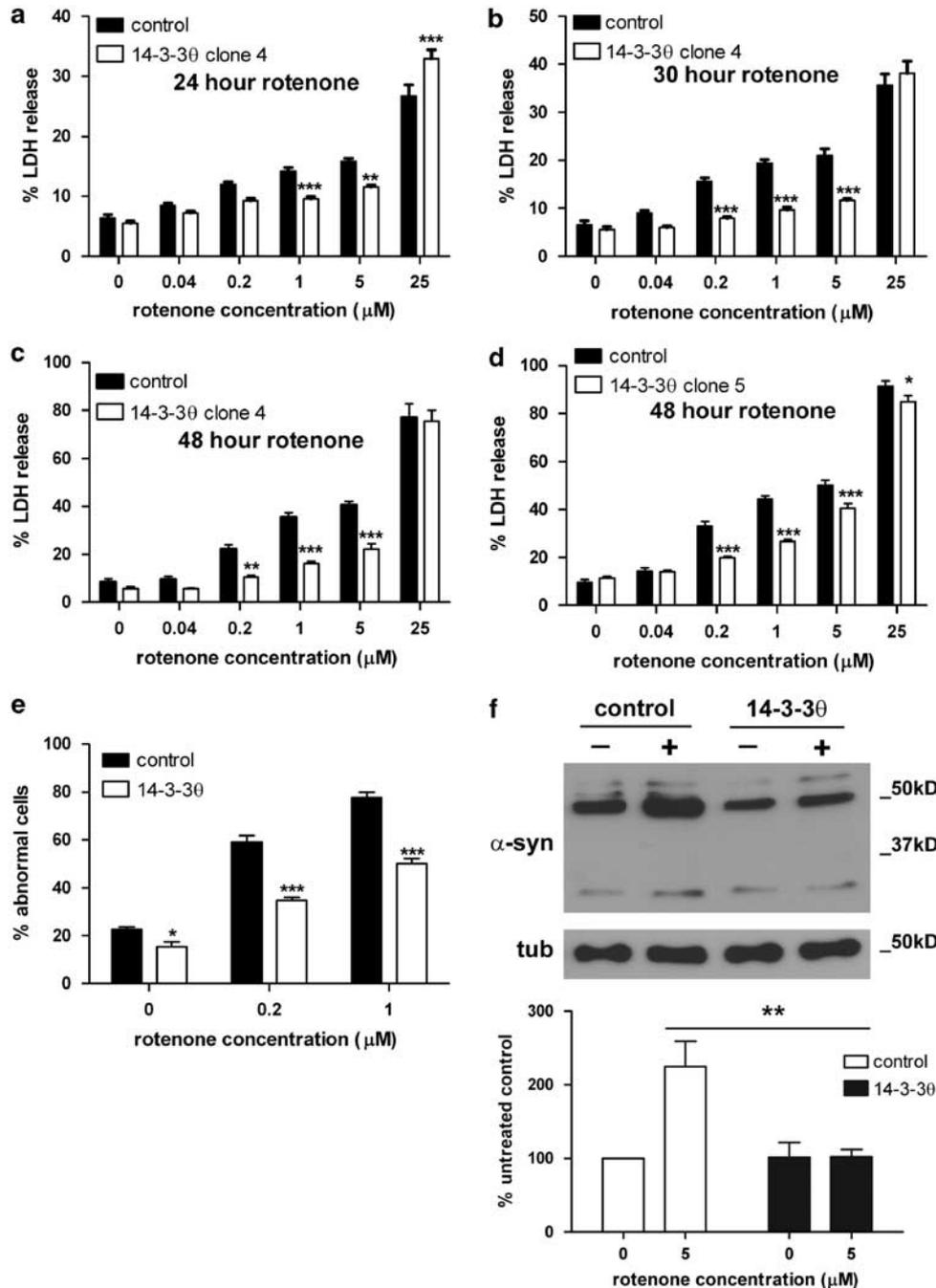
Figure 5 Overexpression of 14-3-3θ protects M17 cells from rotenone toxicity. (**a–c**) Cell lines stably transfected with either 14-3-3θ or empty vector were treated with varying concentrations of rotenone for 24 h (**a**), 30 h (**b**), or 48 h (**c**). Cell death was assayed by LDH release into the culture media. LDH release into media was normalized to total LDH release for each well. The 14-3-3θ-overexpressing line was more resistant to rotenone at several different concentrations compared to control stable cells at all time points tested. (**d**) At the 48 h time point, a second 14-3-3θ-overexpressing stable clone was tested to verify these results. Results reflect 2–3 independent experiments with at least two replicates per experiment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Bonferroni's multiple comparison test). (**e**) To confirm these findings using an alternative cell death assay, we transiently transfected with V5/His-tagged 14-3-3θ construct plasmid into naive M17 cells. Control cells were transfected with GFP. At 24 h after transfection, cells were treated with rotenone at 0, 0.2, or 1 μM for 24 h. Afterwards, cells were fixed and immunostained with an antibody against V5 or GFP, followed by nuclear staining with Hoechst 33342. Nuclei of transfected cells were scored as normal or showing apoptotic changes. Rater was blind to experimental condition. $n = 8$ for each experimental condition. *$P < 0.05$, ***$P < 0.001$ (Bonferroni's multiple comparison test). (**f**) Amount of insoluble α-syn was reduced in 14-3-3θ cells treated with 5 μM rotenone for 24 h compared to control cells. After rotenone treatment, cell lysates were separated into Triton X-100 soluble and insoluble fractions and blotted with an antibody against α-syn. Representative western blot of insoluble fractions is shown. Densitometric quantification of the multiple α-syn bands includes four independent experiments; we quantified the region between ∼30 and ∼70 kDa where all major bands were found. **$P < 0.01$ (Tukey's multiple comparison test). Error bars reflect S.E.M.
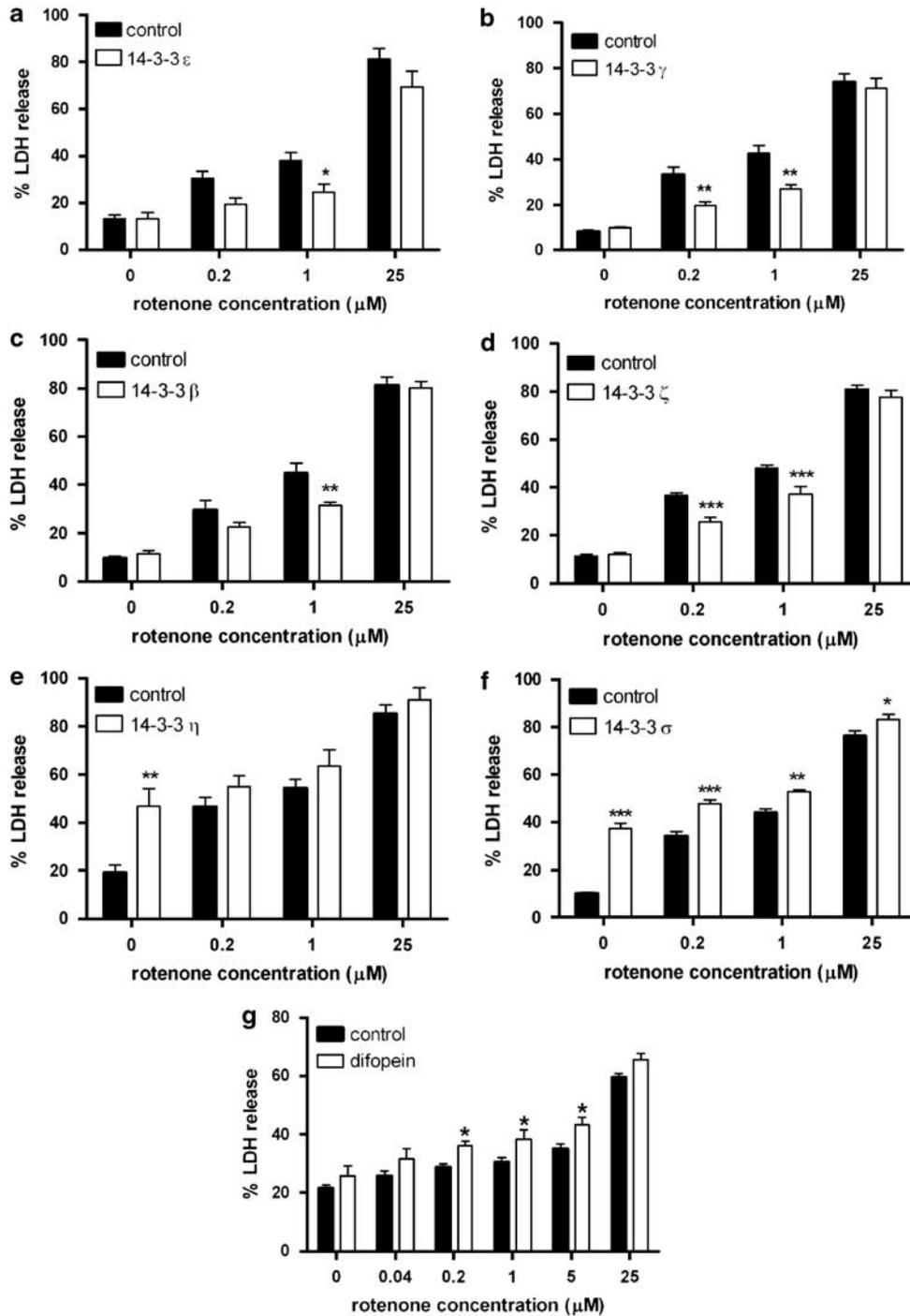
Figure 6 Overexpression of other 14-3-3 isoforms also protects against rotenone toxicity, whereas inhibition of 14-3-3s promotes toxicity. (**a–f**) Cell lines stably transfected with either a 14-3-3 isoform or empty vector were treated with varying concentrations of rotenone for 48 h. Cell death was assayed by LDH release into the culture media. LDH release into media was normalized to total LDH release for each well. Lines overexpressing 14-3-3ε (**a**), 14-3-3γ (**b**), 14-3-3β (**c**), or 14-3-3ζ (**d**) were more resistant to rotenone compared to control stable cells. In contrast, overexpression of 14-3-3η (**e**) and 14-3-3σ (**f**) did not protect against rotenone and showed increased toxicity at baseline. Results reflect 2–3 independent experiments with at least two replicates per experiment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Bonferroni's multiple comparison test). Error bars reflect S.E.M. (**g**) M17 cells were infected with either tetracycline-inducible difopein-EYFP lentivirus or scrambled difopein-EYFP lentivirus. After 2 days of induction by doxycycline, cells were plated and treated with increasing doses of rotenone for 30 h, and cell death was assayed by LDH release. Difopein-expressing cells were more sensitive to rotenone compared to control stable cells. *$P < 0.05$ (least-squares means test). Results reflect three independent experiments with at least two replicates per experiment. Error bars reflect S.E.M.
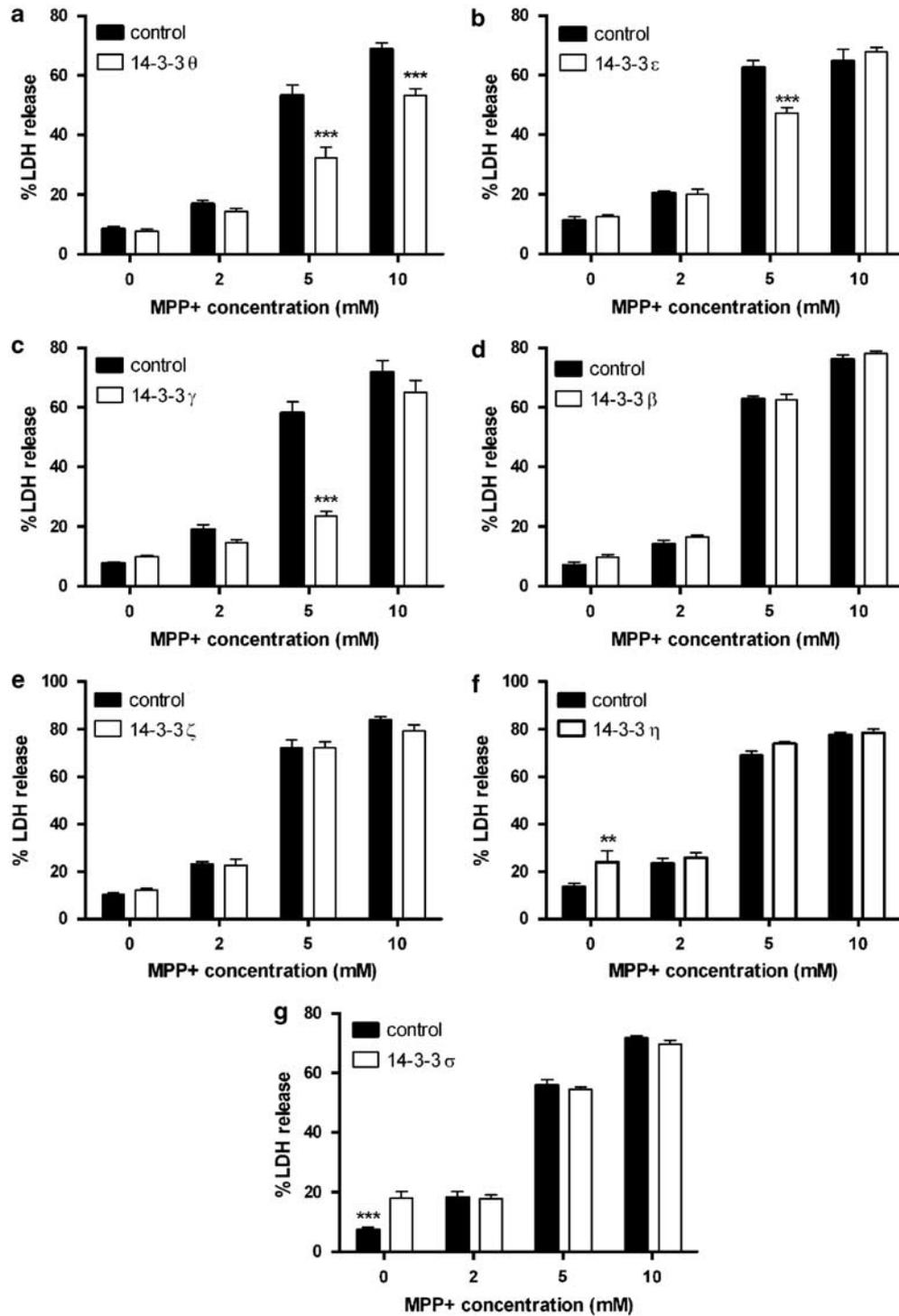
Figure 7 Overexpression of 14-3-3ε, -γ, and -θ protects against MPP$^+$ toxicity. Cell lines stably transfected with either a 14-3-3 isoform or an empty vector were treated with varying concentrations of MPP$^+$ for 24 h. Cell death was assayed by LDH release into the culture media. LDH release into media was normalized to total LDH release for each well. Lines overexpressing 14-3-3θ (**a**), 14-3-3ε (**b**), or 14-3-3γ (**c**) were more resistant to MPP$^+$ compared to control stable cells. In contrast, overexpression of 14-3-3β (**d**), 14-3-3ζ (**e**), 14-3-3η (**f**), or 14-3-3σ (**g**) did not protect against MPP$^+$. Results reflect 2–3 independent experiments with at least two replicates per experiment. **$P < 0.01$, ***$P < 0.001$ (Bonferroni's multiple comparison test). Error bars reflect S.E.M.

alterations in cellular morphology and reduction in GFP expression.[27] We created double transgenic worms in which a particular 14-3-3 isoform and α-syn were overexpressed,

both under the *dat-1* promoter (Figure 8). Three separate transgenic lines were created for each 14-3-3 isoform and analyzed. We focused on 14-3-3ε, -γ, and -θ, the three
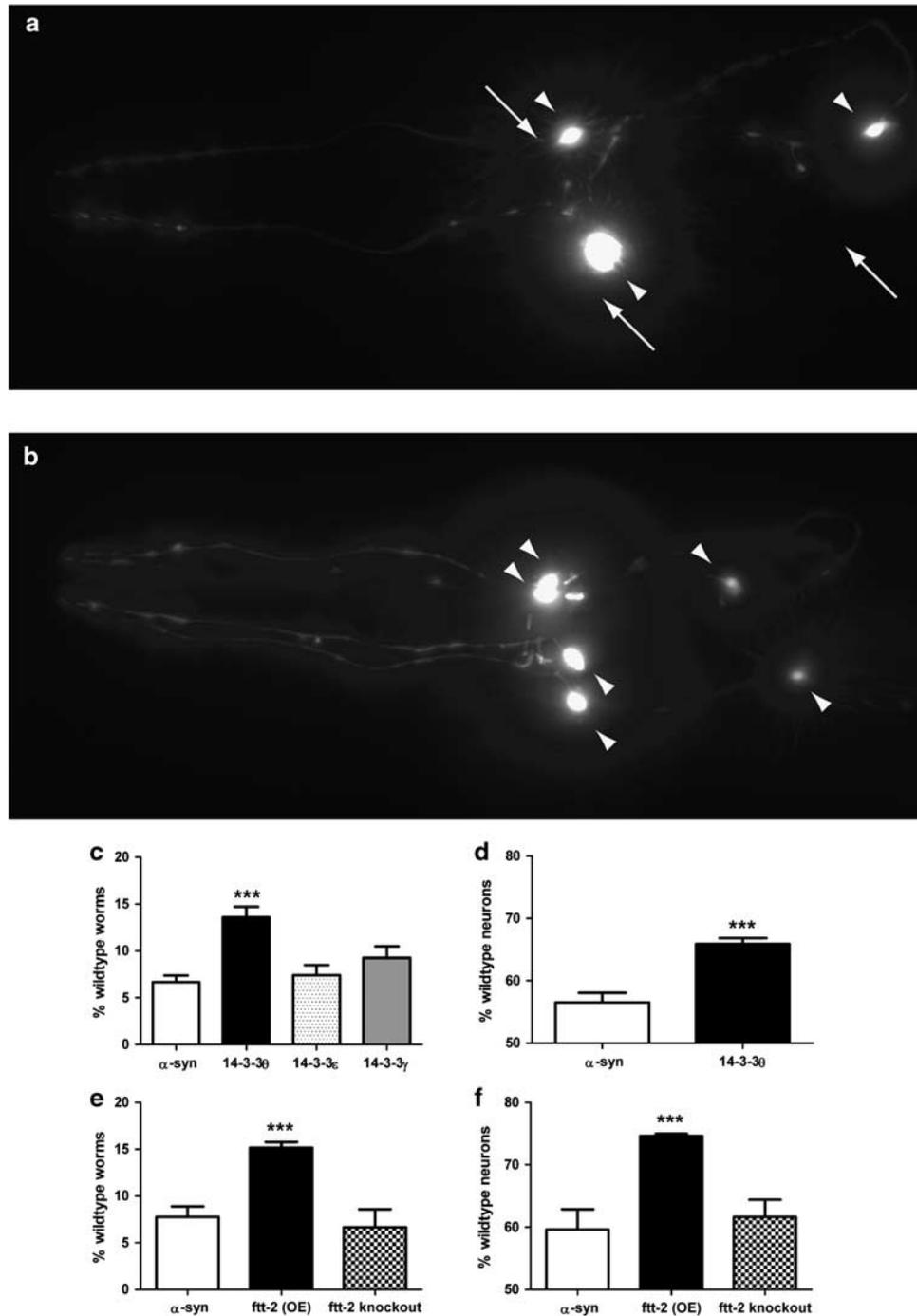
Figure 8 Overexpression of 14-3-3θ protects against dopaminergic cell loss in the α-syn transgenic *C. elegans* worm model. Expression plasmids, P_{dat-1}:: human 14-3-3θ and P_{unc-54}::mCherry, were constructed and microinjected into the gonads of *C. elegans* strain that already expresses α-syn and GFP (P_{dat-1}:: α-syn; P_{dat-1}::GFP) and exhibits age-dependent α-syn-induced degeneration in dopaminergic neurons. Similar double transgenic worms were created to overexpress human 14-3-3ε, human 14-3-3γ, or the worm 14-3-3 homologue *ftt-2*. In addition, a mutant *ftt-2* knockout that overexpressed α-syn was also created. (**a**) α-Syn worm shows loss of dopaminergic neurons (only two CEP neurons and one ADE neuron remain) at day 7. Arrowheads show intact dopaminergic neuron cell bodies. Lined arrows indicate areas where dopaminergic neurons have degenerated. (**b**) Overexpression of 14-3-3θ in the α-syn-overexpressing line protects dopaminergic neurons from α-syn-induced cell death. Arrowheads show six intact dopaminergic neuron cell bodies. (**c**, **d**) 14-3-3θ reduced dopaminergic cell loss, but 14-3-3ε and -γ did not. The numbers of α-syn transgenic worms and α-syn/14-3-3 double transgenic worms that had the full complement of six anterior dopaminergic neurons were scored at day 10 (**c**). The percentage of intact dopaminergic neurons per worm was also scored at day 10 for α-syn and α-syn/14-3-3θ transgenic worms (**d**). (**e**, **f**) Overexpression (OE) of the worm 14-3-3 homologue *ftt-2* also reduced dopaminergic cell death, but *ftt-2* knockout did not enhance neurodegeneration. The percentage of worms that had the full complement of dopaminergic neurons (**e**) and the percentage of intact dopaminergic neurons per worm (**f**) were scored at day 10. For each experiment, three independent transgenic lines were scored, with 30 worms per line analyzed in triplicate experimental trials. Plotted data reflect the average of these three separate lines. ***P < 0.001 (Bonferroni's multiple comparison test). Error bars reflect standard deviation

human isoforms that reduced neurotoxicity in response to both rotenone and MPP$^+$. In worms that overexpressed only α-syn, we found that only 6.67% of the worms had all six anterior dopaminergic neurons present at 10 days, whereas 13.61% of worms that overexpressed human 14-3-3θ along with α-syn had all six dopaminergic neurons at 10 days (Figure 8c; $P<0.001$). Likewise, the average number of intact dopaminergic neurons was significantly higher in the double transgenic 14-3-3θ/α-syn worms compared to α-syn worms (Figure 8d; $P<0.001$). Similar results were seen when worms were analyzed at 7 days (data not shown). Transgenic 14-3-3ε or 14-3-3γ worms did not exhibit a significant change in dopaminergic neurodegeneration (Figure 8c).

We also investigated whether *ftt-2*, the closest *C. elegans* homologue to 14-3-3θ, can protect against α-syn toxicity. Of transgenic worms that overexpress *ftt-2* along with α-syn, 15.19% retained all six dopaminergic neurons, compared to only 7.78% of α-syn worms, at 10 days (Figure 8e; $P<0.001$). Average numbers of intact dopaminergic neurons were significantly higher in the *ftt-2*/α-syn double transgenic worms compared to α-syn worms (Figure 8f; $P<0.001$). We also tested whether knockout of *ftt-2* affected α-syn toxicity. There was no significant difference in dopaminergic cell loss between α-syn worms and α-syn/*ftt-2* knockout worms at 10 days (Figure 8e and f). Semiquantitative RT-PCR was used to confirm lack of *ftt-2* expression (data not shown).

Discussion

Using a diverse set of cellular and animal PD models, we have observed a remarkably consistent neuroprotective effect of several 14-3-3 isoforms. In α-syn transgenic mice, we report prominent mRNA downregulation of 14-3-3ε, -γ, and -θ in the cortex, a site of intense α-syn pathology. We found that these same isoforms reduce α-syn inclusions in neuroglioma cells and attenuate rotenone and MPP$^+$ toxicity in stably transfected neuroblastoma cells. Both human 14-3-3θ and its worm homologue reduced α-syn toxicity in a *C. elegans* model. The effects of the other isoforms were less consistent: 14-3-3β and -ζ protected against rotenone but not MPP$^+$, and 14-3-3η and -σ increased cell vulnerability to nonspecific insults. The consistency of these results suggests commonality in the mechanism of protection in each of these assays, and that the isoforms, although structurally similar, act on different effector systems.

Further support for the specific relationship between 14-3-3ε, -γ, and -θ and PD pathophysiology is provided by studies of Lewy bodies in human post-mortem brain. Using isoform-specific antibodies, we observed 14-3-3ε, -γ, and -θ, as well as 14-3-3ζ in human Lewy bodies, whereas the other isoforms were absent.[28] It should be noted that we were unable to generate a 14-3-3ζ-overexpressing stable line that showed as much overexpression as lines expressing other isoforms. Therefore, it is possible that we failed to observe a broad protective effect of 14-3-3ζ because of insufficient expression. In addition, proteomic analysis of phosphorylation-dependent α-syn interactions revealed that 14-3-3ε, -γ, and -ζ were among those proteins that interact specifically with phosphorylated α-syn.[29]

Although earlier studies have examined the effects of 14-3-3 proteins in PD models, few have considered the differential effects of all the distinct isoforms. One previous study showed that 14-3-3ε overexpression is protective against MPP$^+$ toxicity in PC12 cells but did not evaluate other isoforms.[30] Studies showing interactions between 14-3-3s and α-syn have not clearly defined which isoforms are involved.[11,17] Sato *et al.*[20] provided the most extensive evaluation of different 14-3-3 isoforms in an analysis of 14-3-3 effects on parkin function. 14-3-3η was the only isoform that immunoprecipitated with parkin and inhibited its ubiquitylation activity. We found that 14-3-3η did not affect α-syn aggregation or reduce neurotoxicity induced by rotenone or MPP$^+$ but increased sensitivity to serum deprivation. As 14-3-3η reduces parkin function, overexpression of 14-3-3η could possibly be detrimental to cells by altering protein degradation. This potential mechanism does not explain our 14-3-3σ findings, the other isoform that promoted sensitivity to stressors, as 14-3-3σ was not found to bind parkin or alter its function.[20] 14-3-3σ has been implicated as a tumor suppressor and is inactivated in several cancers;[14,15,31,32] therefore, 14-3-3σ overexpression in cells examined by us could promote sensitivity to stressors like serum deprivation. 14-3-3σ expression is mainly limited to epithelial cells[33] and is unlikely to be relevant to PD pathogenesis.

Currently we do not know the mechanism for the differential 14-3-3 effects observed in our studies. The different isoforms may bind to different ligands that mediate the differential neuroprotective effects. An important ligand may be α-syn. As noted above, a proteomic study showed that 14-3-3ε, -γ, and -ζ interacted with a phosphorylated α-syn peptide, whereas other isoforms were not detected.[29] Here we show that in contrast to the other isoforms 14-3-3ε, -γ, and -θ reduced α-syn inclusion formation. We also observed less insoluble α-syn in 14-3-3θ cells treated with rotenone. Whether aggregation is important to α-syn toxicity, the ability of these isoforms to interact with α-syn may be key to their neuroprotective effects. Alternatively, the differential effects of 14-3-3s may arise from differing abilities to interact with downstream effectors. For example, 14-3-3ε, -θ, and -ζ interact with Bax, whereas 14-3-3β does not.[34] Differential interactions of 14-3-3 proteins with other apoptotic factors could also explain their differential effects.

Although 14-3-3ε, -γ, and -θ showed protection *in vitro*, only human 14-3-3θ overexpression reduced toxicity in the *C. elegans* α-syn model. It is not clear why human 14-3-3ε and -γ were not protective in this worm model. One explanation is that 14-3-3θ is more effective at reducing toxicity. Alternatively, *C. elegans* may not be able to process 14-3-3 isoforms of a different species effectively. Human 14-3-3θ is more homologous with the *C. elegans* 14-3-3 orthologs than are 14-3-3ε and -γ. Indeed, we found that the worm 14-3-3 homologue *ftt-2* was also effective in reducing α-syn toxicity. It is also possible that only human 14-3-3θ can effectively interact with worm apoptotic factors.

In this study, we observed reduced RNA expression of 14-3-3ε, -γ, and -θ in the α-syn transgenic mouse model. Because these are the same isoforms that were protective when overexpressed, this observation raises the possibility that reduction of 14-3-3 expression may contribute to the

progression of PD. We did attempt to examine the effect of reducing 14-3-3θ mRNA levels directly through shRNA inhibition, but found that inhibition of this single isoform had no clear effect on vulnerability to rotenone. Similarly, *ftt-2* knockout in the *C. elegans* model did not promote α-syn toxicity. In both cases, other 14-3-3 isoforms could compensate for the loss of a single 14-3-3 isoform – six others in humans, and one other, *par-5*, in *C. elegans*. We did observe that the broad-spectrum 14-3-3 inhibitor, difopein, had a modest effect on sensitivity to rotenone. There are several potential explanations for the limited magnitude of this effect. First, difopein expression levels could have been limited, as higher levels are likely too toxic for cells to survive in control conditions. Second, given the differential neuroprotective effects of the 14-3-3s, the sum effect of interfering with all isoforms may not be a dramatic increase in toxicity. We are unable to test whether knockout of both *ftt-2* and *par-5* would promote α-syn toxicity in the *C. elegans* model, as loss of *par-5* function is embryonically lethal.[35]

We hypothesize that α-syn can disrupt 14-3-3 function through several mechanisms that may contribute to α-syn toxicity. Previously it has been suggested that the ability of α-syn to sequester 14-3-3s would promote cell death by release of apoptotic factors normally inhibited by 14-3-3s.[11] We propose that α-syn also exerts its effect at a transcriptional level to reduce the expression of functional 14-3-3 proteins: decreased 14-3-3 transcription would compound effect of α-syn on 14-3-3 sequestration by preventing injured neurons from replenishing functional 14-3-3 stores. Functional genomic analysis of our microarray data has shown that genes whose expression is altered in α-syn transgenic mice are predominantly involved in transcription.[13]

Our data point to the potential therapeutic utility of treatments that enhance 14-3-3 function or expression, especially 14-3-3ε, -γ, and -θ. Several compounds that can stimulate 14-3-3 expression have been identified, including peroxisome proliferator-activated receptor-δ (PPARδ) and PPARγ ligands.[36–38] In a mouse stroke model, reduction of infarct volume by rosiglitazone was blocked by siRNA knockdown of 14-3-3ε and restored by increasing 14-3-3ε.[38] The actions of these compounds establish the principle that small molecules can produce potent upregulation of 14-3-3 expression, and point to the possibility of developing a useful human neuroprotective agent that uses this mechanism.

Materials and Methods

Animals. α-Syn transgenic mice originally generated by Masliah *et al.*[39] were bred at Charles River Laboratories (Wilmington, MA, USA) to generate transgenic and wild-type littermates. The use of mice was supervised by the Massachusetts General Hospital Animal Resources Program in accordance with the PHS policy on Humane Care and Use of Laboratory Animals. Mice were killed by CO_2 inhalation. Gender-matched wild-type and transgenic mice were killed at 3 or 9 months of age.

Quantitative PCR. RNA was extracted from the cortex of wild-type and transgenic mice and reverse transcribed into first-strand cDNA using the SuperScript II Reverse Transcriptase Kit (Invitrogen). Primers against each of the human 14-3-3 isoforms were designed using Primer3 (http://frodo.wi.mit.edu). Primers against 14-3-3β (NM_018753) were 5′-aaaggtcccgtgctcatacc-3′ (forward) and 5′-gccgcctca acacattattc-3′ (reverse). Primers against 14-3-3ε (NM_009536) were 5′-ttgggtgttag cttgaggtg-3′ (forward) and 5′-gaggagtcggcaagaatgag-3′ (reverse). Primers against

14-3-3γ (NM_018871) were 5′-tctgtgtcccgcttgtactg-3′ (forward) and 5′-aggcaggcac acttctcaac-3′ (reverse). Primers against 14-3-3η (NM_011738) were 5′-tctagcaaatc caggtgatgg-3′ (forward) and 5′-aggctgatggtgaaggaatg-3′ (reverse). Primers against 14-3-3θ (NM_011739) were 5′-aggagtgacagcacacttgg-3′ (forward) and 5′-gttgcttctg aaaggaaacctc-3′ (reverse). Primers against 14-3-3σ (NM_018754) were 5′-gtctgtcc ttcatcgcagtc-3′ (forward) and 5′-tcctcgttgctcttctgctc-3′ (reverse). Primers against 14-3-3ζ (NM_011740) were 5′-ggctagtgattggaggaaacc-3′ (forward) and 5′-tcatatcg ctctgcctgctc-3′ (reverse). Real-time quantitative PCR was performed as previously described.[13] We used GAPDH (forward primer 5′-tggtgaagcaggcatctga-3′; reverse primer 5′-tgctgttgaagtcgcaggag-3′) to normalize PCR results.

α-Syn inclusion assay. H4 cells were transfected with synphilin, α-syn/synT, and either empty vector control or a 14-3-3 isoform using SuperFect transfection reagent (Qiagen, Germantown, MD, USA), as previously described.[22] At 24 h after transfection, cells were fixed with 4% paraformaldehyde and immunostained with a monoclonal antibody against α-syn (BD Biosciences, San Diego, CA, USA) and a secondary Alexa 488-conjugated goat anti-mouse antibody (Invitrogen). All cells stained for α-syn were scored as positive or negative for α-syn inclusions, with the rater blind to experimental condition.

Creation of stable cell lines. Each 14-3-3 isoform was subcloned into the mammalian expression vector pcDNA3.1/V5-His (Invitrogen). SK-N-BE(2)-M17 cells (ATCC, Manassas, VA, USA) were transfected with each isoform or empty vector using SuperFect, and selection for stably transfected cells was performed in the presence of G418 (Invitrogen). Some 15–20 clones for each isoform were selected for evaluation of 14-3-3 expression by western blotting.

Immunoblotting. Control and 14-3-3 stable M17 cells were sonicated for 10 s on ice in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA)) and centrifuged at $16\,000 \times g$ for 10 min. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Samples were boiled for 5 min in $4 \times$ DTT sample loading buffer (0.25 M Tris-HCl (pH 6.8), 8% SDS, 200 mM DTT, 30% glycerol, Bromophenol Blue), resolved on 15% SDS-polyacrylamide gels, and transferred to PVDF membranes. Blots were blocked in 5% non-fat dry milk in TBST (25 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20) for 1 h, and then incubated overnight with primary mouse monoclonal antibody against V5 (1 : 5000; Invitrogen), mouse monoclonal antibody against 14-3-3θ (1 : 10 000; Abcam, Cambridge, MA, USA), or mouse monoclonal antibody against α-tubulin (1 : 1000; Sigma, St Louis, MO, USA). After three washes in TBST, blots were incubated with HRP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h and then washed in TBST six times for 10 min each. Blots were developed with enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ, USA).

For α-syn blots of stable cell lines, cells were sonicated in lysis buffer (175 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, protease inhibitor cocktail (Roche Diagnostics)) and incubated with 1% Triton X-100 for 30 min. After centrifugation, the supernatant was collected as the Triton X-100 soluble fraction. The Triton X-100 insoluble fraction was prepared by resuspension of the pellet in lysis buffer with 2% SDS. Samples were immunoblotted as described above, using a monoclonal anti-α-syn antibody (1 : 1000; BD Biosciences). Densitometry was used to quantitate the multiple α-syn bands (between $\sim$ 30 and 70 kDa) in the insoluble fraction, which were normalized to tubulin levels.

Immunocytochemistry. Naive M17 cells, control stable cells, and 14-3-3-overexpressing stable cell clones were fixed in 4% paraformaldehyde and permeabilized with Triton X-100. After incubation with 1% normal goat serum, cells were incubated overnight at 4°C with a mouse monoclonal antibody against the V5 epitope tag (Invitrogen), a rabbit polyclonal antibody against 14-3-3ε (Abcam), a mouse monoclonal antibody against 14-3-3θ (Abcam), or a rabbit polyclonal antibody against α-syn (Cell Signaling, Danvers, MA, USA). Following washes in TBS, cells were incubated with cy3-conjugated goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch) for 2 h at room temperature. Nuclei were stained with Sytox Green (Invitrogen).

LDH assay. Cells were grown in pyruvate-free DMEM for a few days before plating in 24-well collagen-treated plates. The following day, cells were treated with varying concentrations of rotenone (0, 0.04, 0.2, 1, 5, or 25 μM; Sigma) or MPP$^+$ (0, 2, 5, or 10 mM; Sigma) in serum-free DMEM for 24, 30, or 48 h. Toxicity was

assayed by LDH release into media using the LDH assay kit (Roche). LDH release into media was normalized to total LDH release for each well.

RNA interference. The 14-3-3θ shRNA pLKO.1 lentiviral constructs created by The RNA Consortium were purchased from Open Biosystems (Huntsville, AL, USA). Packaging of lentiviral shRNA constructs was performed by the UAB Neuroscience NINDS Protein Core (P30 NS47466). Four shRNA constructs were tested for their efficacy in reducing 14-3-3θ protein expression, and two constructs were chosen for use in the rotenone toxicity experiments. M17 cells were infected with the empty vector pLKO.1 virus or one of the two 14-3-3θ shRNA-containing pLKO.1 viruses in the presence of polybrene (Sigma). At 72 h after infection, infected cells were selected for in the presence of puromycin (Mediatech, Herndon, VA, USA). Selected cells were used for rotenone toxicity experiments as described under LDH assay.

Difopein experiments. Difopein-EYFP and scrambled difopein-EYFP were subcloned into the tetracycline-inducible lentiviral vector pSLIK.[26] M17 cells were infected with the difopein-EYFP or control-EYFP viruses in the presence of polybrene. At 72 h after infection, infected cells were selected for in the presence of hygromycin (EMD Biosciences, Gibbstown, NJ, USA). Two days before rotenone toxicity experiments, selected cells were treated with doxycycline (EMD Biosciences) at 10 µg/ml to induce difopein-EYFP or control-EYFP expression.

Generation and analysis of transgenic *C. elegans*. Expression plasmids, P$_{dat-1}$::14-3-3θ, P$_{dat-1}$::14-3-3γ, P$_{dat-1}$::14-3-3ε, and P$_{dat-1}$::ftt-2, as well as a marker, P$_{unc-54}$::mCherry, were constructed by Gateway Technology (Invitrogen) and microinjected into the gonads of *C. elegans* strain UA44 (baln11; P$_{dat-1}$::α-syn, P$_{dat-1}$::gfp) that already expresses α-syn and GFP and exhibits age-dependent α-syn-induced degeneration in the dopaminergic neurons.[27] Three resulting independent transgenic lines for each strain UA113 (baln11; baEx88, P$_{dat-1}$::14-3-3θ, P$_{unc-54}$::mCherry), UA114 (baln11; baEx89, P$_{dat-1}$::14-3-3γ, P$_{unc-54}$::mCherry), UA115 (baln11; baEx90, P$_{dat-1}$:: 14-3-3ε, P$_{unc-54}$::mCherry), and UA131 (baln11; baEx100, P$_{dat-1}$::ftt-2, P$_{unc-54}$::mCherry) were synchronized, grown at 20 °C, and analyzed for neuroprotection at days 7 and 10 (4- and 7-day-old adults). For each trial, 30 worms were transferred onto a 2% agarose pad and immobilized with 3 mM levamisole, and the six anterior dopaminergic neurons (four CEP and two ADE dopaminergic neurons) were examined. In total, 90 animals from each of three 14-3-3θ, 14-3-3γ, 14-3-3ε, and ftt-2, transgenic lines were analyzed (3 lines × 3 trials of 30 animals/trial = 270 total animals scored per 14-3-3 isoform). Worms displaying at least one degenerative change were scored as exhibiting degenerating neurons, as previously described.[27,40] The number of intact neurons per worm was also counted. To study a ftt-2 loss of function mutant, *C. elegans* knockout strain MT14355 (ftt-2(n4426)) was crossed into strain UA44 (baln11; P$_{dat-1}$::α-syn, P$_{dat-1}$::gfp) to generate the isogenic strain UA130 (baln11; ftt-2(n4426)). This strain was synchronized and analyzed as described above. Fluorescence microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with Endow GFP HYQ filter cube (Chroma Technology, Rockingham, VT, USA), and images were captured with a Cool Snap CCD camera (Photometrics, Tucson, AZ, USA) with MetaMorph software (Molecular Devices, Downingtown, PA, USA).

Statistical analysis. GraphPad Prism 5 (GraphPad Inc., La Jolla, CA, USA) and SAS (SAS Inc., Cary, NC, USA) were used for statistical analysis of experiments. Quantitative PCR data were analyzed by two-way ANOVA, followed by *post hoc* least-squares means test. α-Syn aggregation experiments were analyzed by one-way ANOVA, followed by *post hoc* Bonferroni's multiple comparison test. LDH assay experiments were analyzed by two-way ANOVA, followed by *post hoc* Bonferroni's multiple comparison test. α-Syn blots were analyzed by one-way ANOVA, followed by *post hoc* Tukey's multiple comparison test. *C. elegans* data were analyzed by two-way ANOVA, followed by *post hoc* Bonferroni's multiple comparison test.

Conflict of interest

Talene Yacoubian and David Standaert have a pending patent application regarding the use of 14-3-3 proteins in the treatment of neurodegeneration. Sunny Slone, Adam Harrington, Shu Hamamichi, Jennifer Schieltz, Kim Caldwell, and Guy Caldwell declare no potential conflict of interest.

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