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Protein interacting with C kinase (PICK1) is a suppressor of spinocerebellar ataxia 3-associated neurodegeneration in *Drosophila*

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Spinocerebellar ataxia 3 (SCA3) is the most common autosomal dominant ataxia. The disease is caused by an expansion of a CAG-trinucelotide repeat region within the coding sequence of the *ATXN3* gene, and this results in an expanded polyglutamine (polyQ) tract within the Ataxin-3 protein. The polyQ expansion leads to neuronal dysfunction and cell death. Here, we tested the ability of a number of proteins that interact with Ataxin-3 to modulate SCA3 pathogenicity using *Drosophila*. Of 10 candidates, we found four novel enhancers and one suppressor. The suppressor, *PICK1* (*Protein interacting with C kinase 1*), is a transport protein that regulates the trafficking of ion channel subunits involved in calcium homeostasis to and from the plasma membrane. In line with calcium homeostasis being a potential pathway mis-regulated in SCA3, we also found that down-regulation of *Nach*, an acid sensing ion channel, mitigates SCA3 pathogenesis in flies. Modulation of *PICK1* could be targeted in other neurodegenerative diseases, as the toxicity of SCA1 and tau was also suppressed when *PICK1* was down-regulated. These findings indicate that interaction proteins may define a rich source of modifier pathways to target in disease situations.

INTRODUCTION

The most common dominantly inherited ataxia is spinocerebellar ataxia 3 (SCA3). The mutation is the result of a CAG-trinucleotide expansion in the coding region of the *ATXN3* gene, which leads to the expression of a large stretch of glutamines (polyQ) within the protein. There are eight other polyQ diseases, including several of the SCAs (SCA1, 2, 6, 7 and 17) and Huntington's disease. Although the genes responsible for the polyQ diseases appear to be different in amino acid sequence and function, they share pathological hallmarks. For example, this group of diseases is characterized by the formation of polyQ protein aggregates in the nucleus or cytoplasm (1–4). Studies show that the polyQ accumulations sequester proteins involved in the ubiquitin proteasome system (UPS) (5–8). In addition to UPS impairment, it has been proposed that the toxic polyQ protein may impair transcription, mitochondrial function, cytoskeletal transport, genome stability and calcium homeostasis (9). Several therapeutic compounds have been proposed that target protein mis-folding and aggregation, excitotoxic mechanisms and oxidative stress (10,11).

It has become apparent that the polyQ proteins can interact with each other. For example, loss of the *Drosophila ATXN2* homologue, *dAtx2*, mitigates SCA1 and SCA3 pathogenesis, suggesting the possibility of mis-regulation of common molecular pathways (12,13). In addition, a human protein–protein interaction network for 23 different ataxia-causing proteins and 31 ataxia-interacting proteins (14) revealed an interconnected protein network that may prove critical to pathogenesis in humans. Indeed, novel disease interactors were found in the ataxia network, including Puratrophin-1 (pleckstrin homology domain containing, family G), which was independently identified as an interactor of SCA1 (15). One of the genes in the ataxia-interactome, *AFG3L2*, was found to be responsible for SCA28 (16). These findings highlight that the effects observed in neurodegenerative diseases characterized by ataxia may in part be due to disruption of shared or interacting networks.

The use of model organisms has revealed great insight into the key genes that mediate polyQ-associated neurodegeneration, particularly *Drosophila* (8,17). To identify novel and

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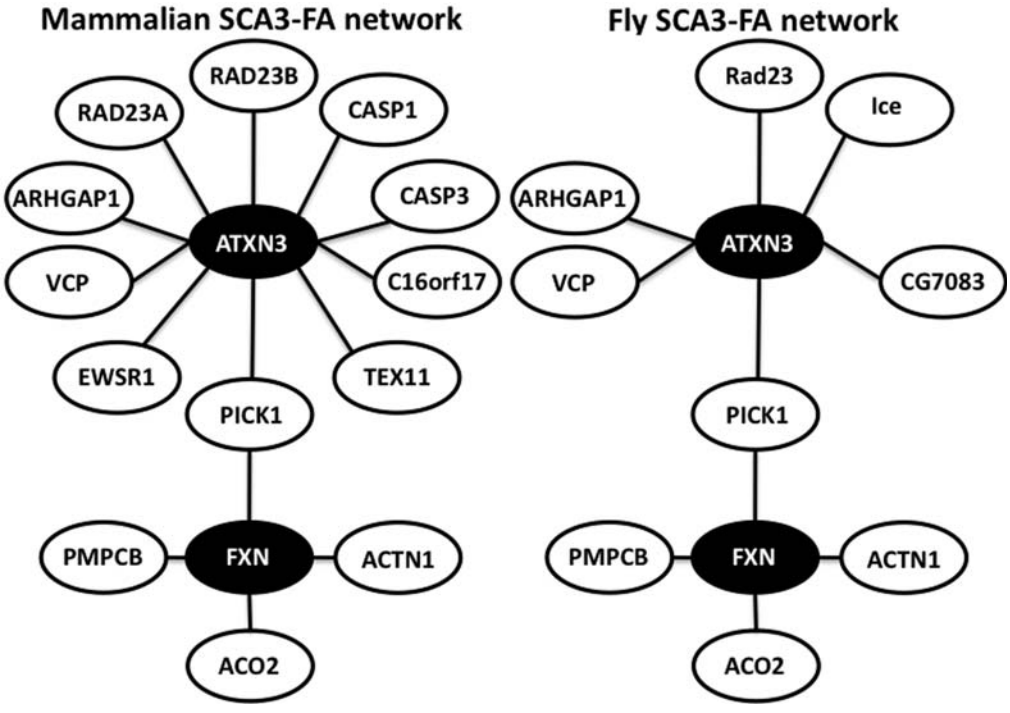

Figure 1. The SCA3-FA network. The ataxia interactome was used to predict protein interactors of Ataxin-3 in *Drosophila* (14). Out of the 10 direct interactors of Ataxin-3, 8 have a clear sequence counterpart in *Drosophila*. Ataxin-3 is one node away from frataxin, the protein responsible for FA. Due to the histopathological similarities between these two diseases, we chose to include the FA-network in our analysis.

common interactors of ataxia, we tested a number of proteins that have been shown to interact with and are predicted to be one to two proteins away from direct interaction with the Ataxin-3 protein in the ataxia interaction network (14). Our studies reveal that the network could be used to successfully predict genetic modifiers of pathogenesis. Our data define *PICK1* (*Protein interacting with C Kinase 1*) as a modifier of SCA3 pathogenesis, as well as Ataxin-1 and tau-associated toxicity. These data suggest that *PICK1*-associated pathways may be neuroprotective and a potential target for neurodegenerative diseases.

One proposed mechanism of polyQ toxicity is mitochondrial dysfunction (19–21). Oxidative stress has been shown to cause an increase in the nuclear accumulation of both pathogenic and normal Ataxin-3 protein, indicating that oxidative stress may be an important factor that influences the pathogenesis of SCA3 (22). One of the interactors, PICK1, connects Ataxin-3 with the mitochondrial protein Frataxin, which is responsible for Friedreich's ataxia (FA) (23). FA and SCA3 share pathology in that the purkinje cells are preserved, unlike SCA1 and SCA2, and both SCA3 and FA affect the dentate nuclei and the dorsal nuclei of Clarke (24). We therefore included the FA network in the analysis of SCA3 pathogenesis.

RESULTS

The SCA3–Friedreich's ataxia network

The ataxia interactome was a project based on 54 proteins, of which 23 are ataxia-causing genes and 31 are genes that were interactors of or paralogues of ataxia-causing genes. Yeast two-hybrid analysis using these 54 genes as bait revealed 561 interacting proteins. Further database mining with the 615 proteins extended the network to 3607 that could be involved in the pathogenesis of ataxia (14). We concentrated on the proteins identified as direct interactors of Ataxin-3. Of the 10 proteins, 9 have clear sequence orthologues in the *Drosophila* genome: Rad23A and Rad23B both share sequence similarity to Rad23, and Caspase 1 and Caspase 2 share sequence homology with Ice (Fig. 1). We excluded VCP from our analysis because it is an established modulator of SCA3 pathogenesis (18).

The effect of the FA network on SCA3 pathogenesis

To investigate whether the FA network could genetically interact with SCA3 toxicity, we first tested the effect of down-regulating the *frataxin homolog* (*fh*) gene. Expression of the polyQ domain of mutant Ataxin-3 (SCA3trQ78) in all tissues of the fly eye using the eye-specific *gmr-GAL4* driver results in degeneration of the external eye (Fig. 2A). To test the effect of *fh*, and additional FA-network genes on SCA3-associated toxicity, we co-expressed SCA3trQ78 with RNAi lines directed to *fh*, *Aconitase* (*Acon*), *CG3731* and *α-actinin* (*Actn)* in the fly eye. We consistently saw an enhancement with two independent RNAi lines directed to *fh*, with RNAi lines directed to *Acon* (a protein involved in the tricarboxylic acid cycle) and with *CG3731* (which in mammals cleaves the leader peptides from proteins transported into the

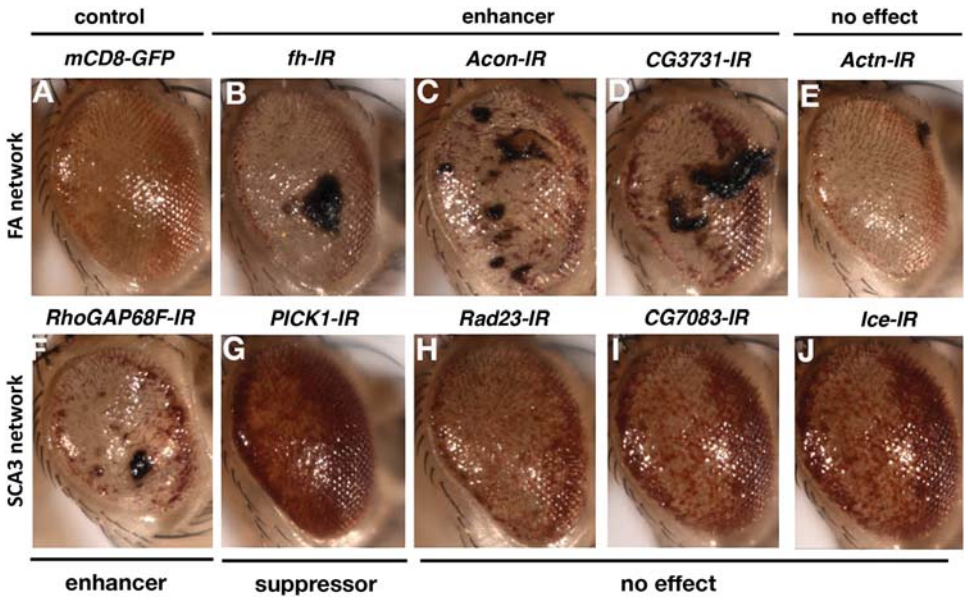

Figure 2. SCA3 candidate modifiers. (**A**) Expression of strong SCA3trQ78 causes degeneration of the external fly eye. Genotype: *w; gmr-GAL4, UAS-SCA3trQ78/UAS-mCD8-GFP.* (**B**–**E**) Genes of the frataxin network. Genotypes: *w; gmr-GAL4, UAS-SCA3trQ78* in trans to the alleles indicated. (B–D) Genes of the frataxin network that enhance the SCA3trQ78 degenerate eye phenotype. (E) Reduction of *Actn,* a frataxin network protein, has no effect on the SCA3trQ78 degenerate eye phenotype. (**F**–**J**) Genes of the SCA3 network. Genotypes: *w; gmr-GAL4, UAS-SCA3trQ78* in trans to the alleles indicated. (F) *RhoGAP68F* when knocked-down enhances the SCA3trQ78 degenerate eye phenotype. (G) Reduction in *PICK1* suppresses the SCA3trQ78 eye phenotype. (H–J) Genes of the SCA3 network, when knocked-down, that have no effect on the SCA3trQ78 degenerate eye phenotype.

mitochondria) (Fig. 2B–D). The modulation was specific as the expression of the *fh* and *Acon* RNAi transgenes with *gmr-GAL4* alone had no effect, whereas expression of the *CG3731* RNAi transgene alone produced a very mild disruption of the external eye (Supplementary Material, Fig. S1). Down-regulation of *Actn*, an actin-binding protein, had no effect on SCA3trQ78 degenerative eye phenotype (Fig. 2E). These data suggest that the down-regulation of components of the mitochondria can enhance SCA3 pathogenesis.

To determine whether these modifiers are dosage-sensitive regulators, we tested whether up-regulation of components of the frataxin network could mitigate SCA3trQ78 pathogenesis. *UAS-fh* and *UAS-Acon* were co-expressed with SCA3trQ78. We found no modification of the external or internal retinal morphology (Supplementary Material, Fig. S2). These data suggest that although down-regulation of these genes that influence mitochondrial function can enhance SCA3 pathogenesis, up-regulation of these components does not mitigate toxicity indicating that other players in this process may be the dose-sensitive regulators of this interaction.

RNAi knockdown of Ataxin-3 interactors reveals new regulators of pathogenesis

To identify additional genes involved in the pathogenesis of SCA3, we concentrated on the proteins that had been identified to directly interact with the Ataxin-3 protein (14) and knocked-down the expression of those genes by RNAi. This revealed that the reduction in *RhoGAP68F* expression enhanced the eye phenotype of SCA3trQ78 (Fig. 2F). *RhoGAP68F*, the *Drosophila* counterpart of the human gene *ARHGAP*, is a GTPase activating protein that negatively regulates Rho GTPase (25). No phenotype was observed when the *RhoGAP68F* transgene was expressed with *gmr-GAL4* alone (Supplementary Material, Fig. S1).

We identified one gene, *PICK1* (protein interacting with C kinase 1), that, when knocked-down, suppressed the external eye degeneration of SCA3trQ78 (Fig. 2G). Real-time polymerase chain reaction (PCR) analysis was performed on flies globally reducing *PICK1* with the *daughterless-GAL4* (*da-GAL4*) driver. The *da-GAL4* driver is expressed in all tissues; this allowed us to determine the efficiency of the knock-down without dilution of wild-type gene levels from tissues not expressing the RNAi transgene. Real-time PCR showed that the *PICK1* RNAi line reduced the *PICK1* mRNA level to below 20% of the control (Fig. 3C). The internal retinal structure of flies co-expressing SCA3trQ78 and the RNAi transgene to *PICK1* also revealed mitigated degeneration (Fig. 3A). Suppression was confirmed with an independent *PICK1* RNAi line, a deficiency line and heterozygous loss of *PICK1* (Supplementary Material, Fig. S3).

To further test the suppression of SCA3trQ78 upon down-regulation of *PICK1*, we also examined the loss of photoreceptor neuronal integrity with knockdown of *PICK1* by RNAi or heterozygous loss of endogenous *PICK1* (26) using an adult-onset assay directing the pathogenic polyQ protein to the photoreceptor neurons. Both of these situations significantly suppressed the degeneration of photoreceptor neurons (Fig. 3D), indicating that the reduction in *PICK1* is neuroprotective in the SCA3trQ78 fly model both with developmental toxicity and in the adult stages.

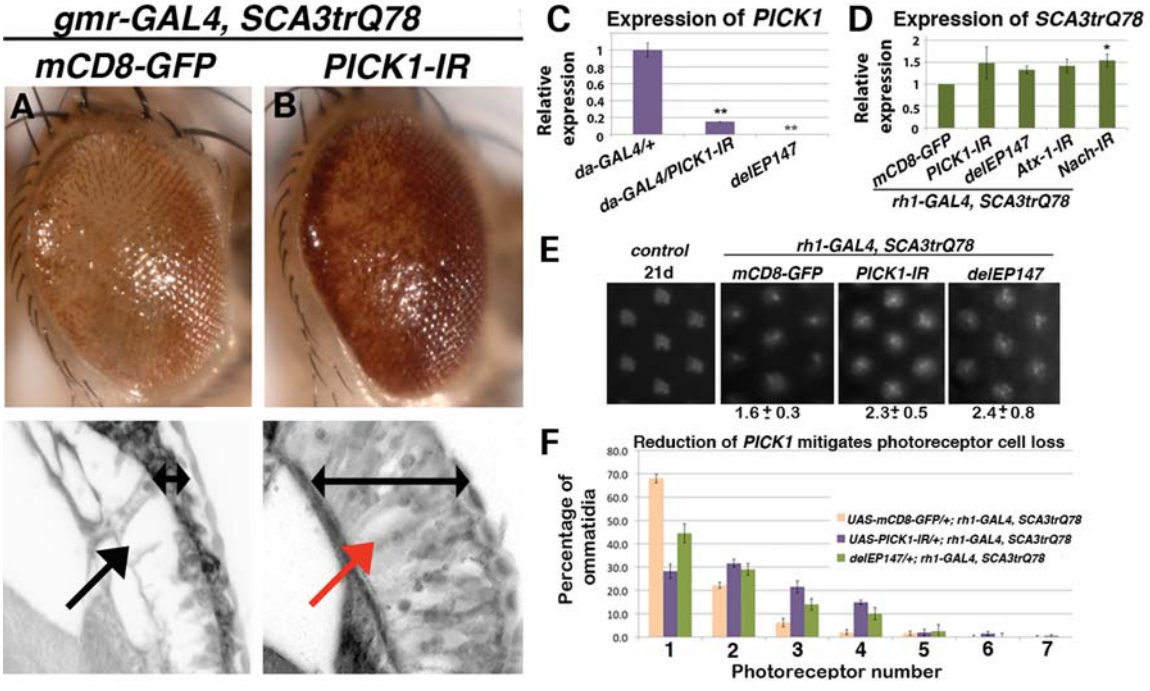

Figure 3. Reduction in *PICK1* is neuroprotective in SCA3. (**A**) Expression of SCA3trQ78 causes external and internal degeneration, with pigment loss and disintegration of retinal structure. Genotype *w; gmr-GAL4, UAS-SCA3trQ78/UAS-mCD8-GFP.* Black arrow indicates loss of tissue, and double-headed arrow indicates retinal tissue width. (**B**) Knockdown of *PICK1* gene expression suppresses the degeneration of the SCA3trQ78 external eye and deterioration of the retinal structure. Genotype *w; gmr-GAL4, UAS-SCA3trQ78/UAS-PICK1-IR.* The red arrow indicates preservation of retinal tissue, and double-headed arrow indicates retinal tissue width. (**C**) Global expression of *UAS-PICK1-IR* with the *daughterless-GAL4* (*da-GAL4*) driver causes a consistent knock-down of *PICK1* expression compared with the control, mean ± SEM is shown, $^{*}P < 0.001$. (**D**) The suppression of the SCA3trQ78 degenerate eye phenotype is not due to a reduction in SCA3 transgene expression. One-way analysis of variance (ANOVA) was performed followed by a Tukey's post-test, $^{*}P < 0.05$, mean ± SEM. (**E**) Photoreceptor neural degeneration is suppressed by *PICK1* reduction. Photoreceptor neurons were counted in eyes of flies by optical neutralization. Representative images of each genotype are shown and the mean ± SEM number of ommatidia is indicated below each image. An aged-matched wild-type control shows the expected non-degenerated ommatidial structure with seven photoreceptors. Data are 10–20 ommatidia of at least 10 flies per genotype, repeated three independent times. (**F**) The distribution of ommatidia in female adults at d21. Expression of SCA3trQ78 in the photoreceptor neurons with the *rhodopsin-1-GAL4* (*rh1-GAL4*) driver results in a severe loss in the number of photoreceptor neurons at day 21. Reduction in *PICK1*, either by the co-expression of the *UAS-PICK1* RNAi transgene or by heterozygous loss of endogenous *PICK1*, suppresses the photoreceptor loss at day 21, $^{*}P < 0.001$, mean ± SEM.

Loss of *PICK1* expression has some effect to reduce insoluble SCA3trQ78 protein accumulation

The formation of polyQ aggregates is a hallmark of the polyQ diseases. In SCA3, the aggregates form insoluble nuclear inclusions (NIs). Expression of SCA3trQ78 in differentiated photoreceptor neurons with the adult-onset photoreceptor driver, *rhodopsin-1-GAL4* (*rh1-GAL4*), allowed us to examine degeneration slowly over time in the adult, and is more sensitive for analysis of changes in protein levels. Expression of SCA3trQ78 in adult photoreceptor neurons results in the detection of insoluble SCA3trQ78 by d3 (Fig. 4A). Co-expression of the RNAi transgene to *PICK1* or heterozygous loss of endogenous *PICK1* reduced the amount of insoluble SCA3trQ78 and increased the amount of soluble protein, such that the ratio of soluble protein (presented as amount of soluble protein relative to tubulin loading control) was significantly increased (Fig. 4B). At d3, the formation of NIs can be observed in the retina of flies expressing SCA3trQ78, with the *rh1-GAL4* driver (Fig. 4C). Reduction in *PICK1* with the RNAi transgene caused a change in the appearance of the accumulations, such that they appeared less compact, and a trend for fewer inclusions was observed (Fig. 4C–E).

To test the broader role of *PICK1* to modulate neurodegenerative-associated proteins, we then tested the ability of *PICK1* to affect Tau toxicity. Mutation in the microtubule-binding protein, Tau, leads to the formation of insoluble, hyperphosphorylated-Tau positive neurofibrillary tangles in Alzheimer's disease (27–29). We expressed a mutant form of human Tau, hTau.R406W, with either the *PICK1* RNAi transgene or with heterozygous loss of endogenous *PICK1*. These experiments showed that the reduction in *PICK1* suppressed the toxicity of hTau.R406W (Fig. 4F). Heterozygous loss of *PICK1* did not suppress the Tau.R406W as well as the RNAi line (Fig. 4E); however, *PICK1* in the heterozygous condition is anticipated to be ~50%, whereas the RNAi line directed to *PICK1* reduced the levels of *PICK1* to 20% of the wild-type (Fig. 3C). These data indicate that down-regulation of *PICK1* activity may be protective in a number of different neurodegenerative situations.

A reduction in *Nach* expression mitigates SCA3 pathogenicity in flies

PICK1 is known to act as a transport protein that traffics the GluR2 subunit away from the plasma membrane and the acid sensing ion channels (ASICs) to the plasma membrane

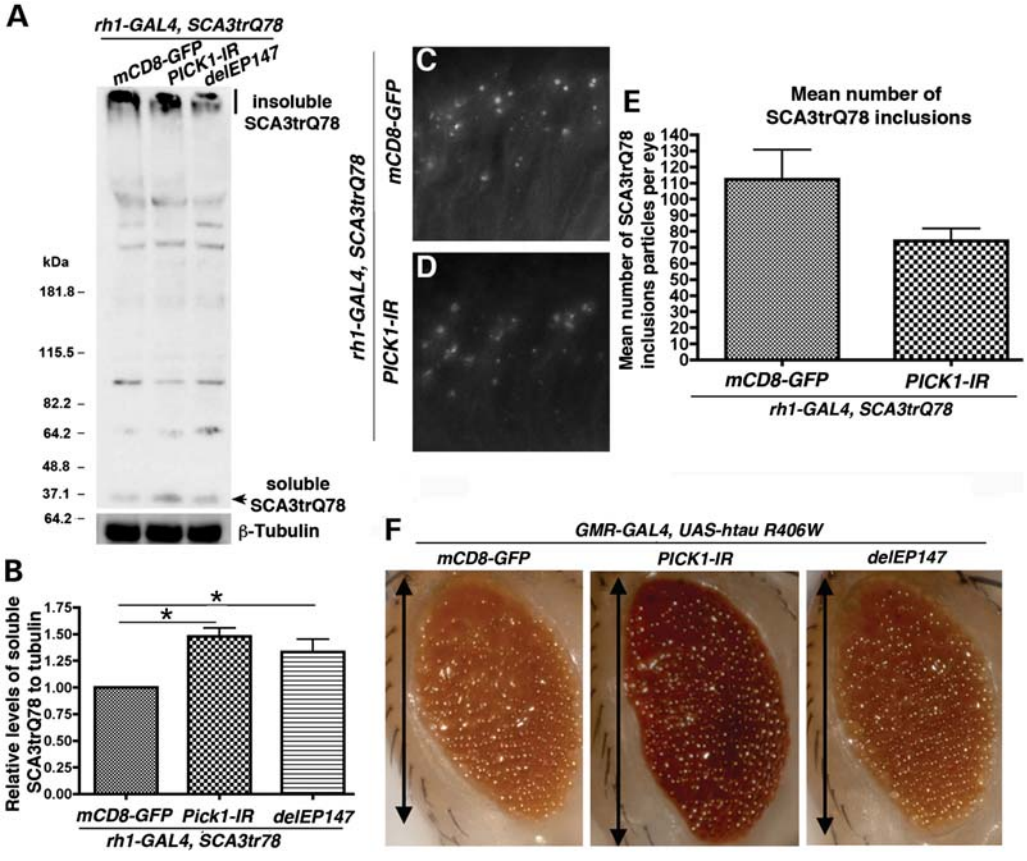

Figure 4. Reduction in *PICK1* expression in the SCA3trQ78 eye leads to less insoluble SCA3 protein. (**A**) Western blot detecting SCA3trQ78 with anti-HA shows that reduction in *PICK1* expression decreases the amount of insoluble SCA3trQ78 protein, present in the stacking gel, and increases the amount of soluble SCA3trQ78 protein. Genotype *w; rh1-GAL4, UAS-SCA3trQ78* in trans to indicated alleles. (**B**) Quantification of the mean ± SEM of six independent repeats showing the ratio of soluble protein relative to the control strain (normalized to tubulin). One-way ANOVA followed by a Tukey's test, $^{*}P < 0.05$. (**C** and **D**) Horizontal cryosections of flies at d3 stained with anti-HA to detect SCA3trQ78. (C) Control flies showing the accumulation of insoluble SCA3trQ78. Genotype *w; UAS-mCD8-GFP /+; gmr-GAL4, UAS-SCA3trQ78+*. (D) Flies with reduced *PICK1* expression show a slight reduction in the accumulation of the SCA3trQ78 protein. Genotype *w; gmr-GAL4, UAS-SCA3trQ78/UAS-PICK1-IR* (**E**) SCA3trQ78 inclusions were quantified in cryosections from animals expressing SCA3trQ78 compared with those expressing the disease protein but now with reduced *PICK1* levels, mean ± SEM. (**F**) Flies expressing hTau.R406W in the eye show a degenerate, reduced eye. Reduction in *PICK1* suppresses the small eye. The *PICK1* deletion line *delEP147* did not suppress the small eye phenotype as robustly as the RNAi line, although the RNAi line reduces PICK1 expression to a much greater effect than the deletion. Genotype: *w, gmr-GAL4; UAS-htau.R406W* in trans to indicated alleles. Double-headed arrow indicates length of fly eye.

(30–33). In both of these situations, there is an increase in the influx of calcium ions, and under conditions of traumatic neuronal injury and ischemia, this leads to an increase in cell death (33–37). Interestingly, calcium excitotoxicity has also been proposed to be involved in the pathogenesis of a number of neurodegenerative diseases, including SCA3, SCA1 and Huntington's disease, and suppression of the mammalian *ACCN2* gene (an ASIC) suppresses Huntington's disease-associated pathology in cells (38–43). These data raised the hypothesis that a reduction in ion channels, and their associated effects on excitability, may also suppress SCA3trQ78 toxicity in flies.

PICK1-mediated transport of ion channels involved in glutamatergic signaling is dependent upon PKCα (30,31,32). We found that inhibition of or a reduction in PKCα failed to suppress SCA3trQ78 toxicity (Supplementary Material, Fig. S4). In addition, the main neurotransmitter in the fly eye is histamine. Together, this suggests that modulation of glutamatergic signaling in the SCA3trQ78 fly would have no effect. We therefore focused on the ASICs. Intriguingly, knockdown of one ASIC gene in the fly eye, *Nach*, suppressed the eye degeneration of SCA3trQ78 (Fig. 5A and B), whereas knockdown of a second ASIC, *pickpocket* (*ppk*), only partially suppressed the retinal degeneration (Supplementary Material, Fig. S5). There are 16 different ASIC genes in the fly genome. We tested all RNAi lines available to these genes (14 genes) but did not see suppression upon reduction in any additional genes (data not shown). It could be that the reduction in a combination of two or more of these other ASIC proteins may be required to offer protection in the SCA3 model, or that *Nach* is the primary ASIC mediating the suppression of SCA3trQ78 in the retina.

SCA3 shares common pathogenic pathways to SCA1

Several lines of evidence suggest that ataxia proteins share common molecular pathways. For example, in flies reduction in *dAtx2* suppresses SCA3- and SCA1-induced pathogenesis and up-regulation of the normal Ataxin-3 protein can suppress Atx1Q82 (SCA1) degenerative phenotypes (12,13,44). We

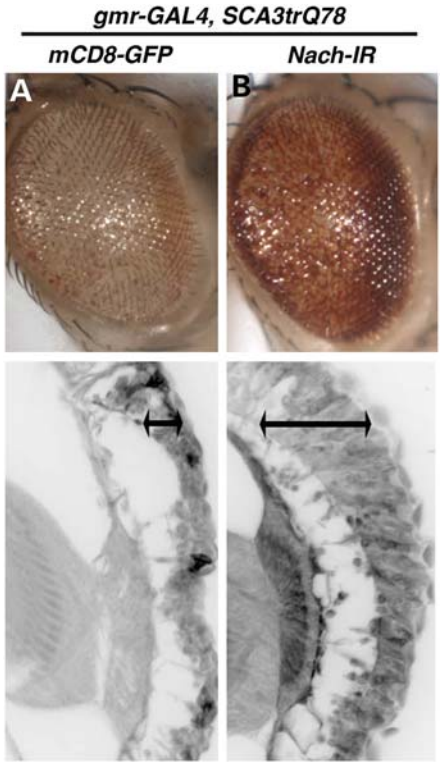

Figure 5. Reduction in *Nach*, an ASIC, mitigates SCA3 pathogenesis. (**A**) Control strain showing degeneration of the external eye and of the retina. Genotype: *w; gmr-GAL4, UAS-SCA3trQ78/UAS-mCD8-GFP.* Double-headed arrow indicates retinal tissue width. (**B**) Co-expression of an RNAi transgene to *Nach*, an ASIC, suppresses the external internal degeneration induced by SCA3trQ78. Genotype: *w; gmr-GAL4, UAS-SCA3trQ78/UAS-Nach-IR.* Double-headed arrow indicates retinal tissue width.

therefore tested whether there was also an interaction between the normal *Atx-1* gene and SCA3. To do this, we down-regulated *Drosophila Atx-1* in the SCA3trQ78 fly. This revealed a dramatic suppression of retinal degeneration (Fig. 6A and B), further suggesting that SCA1 and SCA3 may share pathogenic pathways. We therefore went on to test the effect of down-regulating *PICK1* in the SCA1 fly model. Similar to expression of pathogenic SCA3, expression of the Ataxin-1 protein bearing 82 polyglutamine repeats (hAtx1Q82) causes degeneration of the external and internal retina (Fig. 6C) (41). This degeneration was strongly suppressed by reduction in *PICK1* (Fig. 6D); however, in this situation, reduction in *Nach* did not suppress hAtx1Q82-associated degeneration (data not shown). Thus, degeneration by both pathogenic hAtx1 and SCA3 is mitigated by *PICK1* reduction, although ASIC proteins appear distinct as *Nach*-mitigated SCA3 pathogenicity, whereas it had no effect on SCA1 toxicity.

DISCUSSION

The polyQ diseases share several pathogenic cellular mechanisms, all of which result in neuronal toxicity and neuronal degeneration. Ataxia occurs in several of the polyQ diseases, such as the spinocerebellar ataxias, due to the neuronal loss in the cerebellum and other motor control regions. A human protein–protein interaction network of ataxia-associated proteins revealed an interconnected network, suggesting that the ataxia-causing genes, which share phenotypic features, also share common molecular pathways (14). Using an RNAi approach, we disrupted some of the major molecular players proposed to be involved in interactions with Ataxin-3 or this polyQ protein network: mitochondrial dysfunction, cytoskeletal transport, genome stability or calcium homeostasis (9). Our studies support the idea that mitochondrial dysfunction and calcium homeostasis may be critical. Moreover, although this network is based on the normal proteins, and not the mutant proteins, our data suggest the network can modulate pathogenicity of the proteins associated with human disease *in vivo*.

Disrupting mitochondrial function by reducing the expression of three distinct mitochondrial genes, *fh*, *Acon* and *CG3731*, enhanced SCA3 pathogenesis. These genes, however, do not appear to be rate-limiting because up-regulation of *fh* and *Acon* did not suppress degeneration. Thus, neurons expressing pathogenic SCA3 are susceptible to oxidative stress; however, up-regulation of components of the frataxin network with drugs such as erythropoietin may not be of benefit to SCA3 patients as the processes influenced by these genes is not dose-sensitive and rate-limiting in an up-regulation manner. The key nodes of these networks that are dose-sensitive would be important to reveal and target.

Two of the six proteins predicted to interact with the Ataxin-3 protein also genetically modified SCA3 disease pathogenesis in the fly. Reduction in the gene encoding cdc42 GTPase activating protein, *RhoGAP68F,* enhanced pathogenesis, whereas loss of *PICK1* suppressed toxicity, indicating that reduction in *PICK1* gene expression is neuroprotective. Recently, a small molecule inhibitor targeted to the PDZ (post synaptic density protein-95, *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein) domain of PICK1 was identified (45,46). It is therefore plausible to test the effect of inhibiting PICK1 function with small molecule inhibitors in the treatment of SCA3 and in other SCA3 model systems (39).

PICK1 is known to transport subunits of ion channels to and from the plasma membrane (30–33). The reduction in mammalian PICK1 function reduces excitotoxicity which has led to great interest into therapeutically targeting PICK1 for ischemia (36). The mutant Ataxin-3 protein has also been found to associate and activate calcium release from the calcium transporter $InsP_3R1$ (39). With this in mind, we investigated whether other proteins involved in calcium homeostasis could suppress SCA3 toxicity. This approach revealed that reduction in *Nach,* an ASIC, recapitulated the suppression observed with the reduction in *PICK1*, suggesting that this channel may be involved in the toxicity of the pathogenic SCA3 protein.

Our work also revealed that a reduction in the expression of the *Drosophila* homologue of *ATXN1*, *Atx-1*, in the SCA3 fly model mitigated disease pathogenesis, suggesting that SCA1 and SCA3 pathogenesis may share more molecular pathways than previously appreciated. These studies indicate that therapeutics directed at SCA1 may be of benefit to SCA3 due to overlapping functional pathways. We also tested whether *PICK1* reduction could mitigate hAtx1Q82 toxicity. This result was unanticipated, as PICK1 had not been predicted to bind to the Ataxin-1 protein in the ataxia interactome

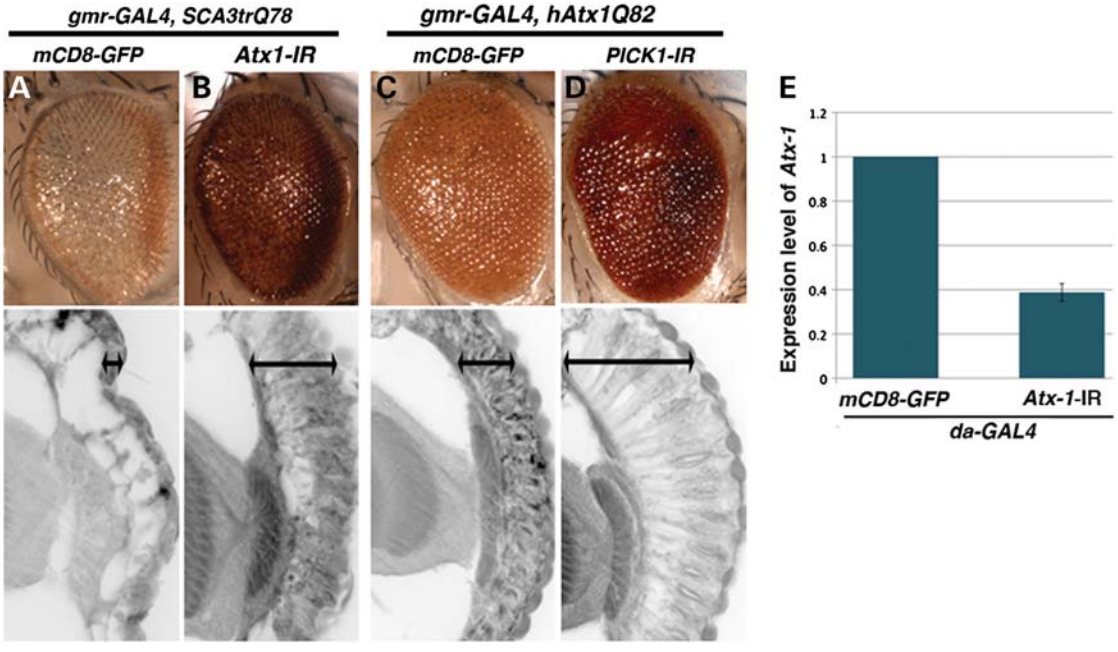

Figure 6. SCA1 shares common pathogenic pathways to SCA3. (**A**) Control strain shows degeneration of the external eye and of the retina. Genotype: *w; gmr-GAL4, UAS-SCA3trQ78/UAS-mCD8-GFP*. (**B**) Co-expression of an RNAi transgene directed to *Atx-1* suppresses the SCA3 degeneration. *w; gmr-GAL4, UAS-SCA3trQ78/UAS-Atx-1-IR*. (**C** and **D**) Reduction in *PICK1* gene expression suppresses hAtx1Q82 degeneration. (C) Expression of the human Ataxin-1 protein with an expansion of Q82 causes degeneration of the external eye and of the retina. Genotype: *w-; UAS-mCD8-GFP/+; gmr-GAL4 (YHIII), UAS-hAtx1-Q82*. (D) Co-expression of the RNAi transgene directed to *PICK1* suppresses the SCA1-associated degeneration. Genotype*: w-; gmr-GAL4, UAS-Atx1 Q82/UAS-PICK1-IR*. Double-headed arrow indicates retinal tissue width. (**E**) Global expression, with the *da-GAL4* driver, of the RNAi transgene directed to *Atx-1* consistently reduces endogenous *Atx-1* gene levels (mean ± SEM, $P < 0.03$).

(14). Studies in flies have revealed that the ataxia-causing proteins share molecular and pathogenic activities, since *Drosophila dAtx-2* interacts genetically with both hAtx-Q82 and SCA3 (12,13). Given that we have found that reduction in *PICK1* can suppress both SCA3 and hAtx1-Q82 pathogenesis in flies, *PICK1* may be involved in the shared pathogenic pathways.

Our data suggest that protein interaction networks may be rich sources of genomic modification pathways for pathogenic proteins, and vice versa—such modifiers may be key candidates to test for direct interactions with pathogenic proteins of interest. Finally, our studies suggest that excitotoxic pathways may be of interest to attack not only in ischemic situations, but also in neurodegenerative situations.

MATERIALS AND METHODS

Drosophila stocks and crosses

Fly lines were grown in standard cornmeal molasses agar with dry yeast at 25°C. Transgenic lines for SCA3trQ78 have been described previously (44,47,48). We used UAS-mCD8-GFP as a control transgene to co-express a second transgenic line, when comparing any experimental UAS-transgenic lines to the normal degeneration induced by the SCA3 toxic protein. RNAi lines included: PICK1 [P(KK109273)VIE-260B], RhoGAP68F [P(KK102738)VIE-260B], Rad23 [P(KK107826)VIE-260B], Ice [w1118; P(GD12284)v28065 and w1118; P(GD12284)v28064/TM3], CG7083 [P(KK101270)VIE-260B], CG3731 [P(KK108539)VIE-260B], Acon [P(KK100999)VIE-260B] and Actn [w^{1118}; P(GD1354)v7760 and w^{1118}; P(GD1354)v7762] from the VDRC stock center (49). RNAi lines were also obtained to PICK1 (line TRiP.JF01199), Atx-1 (line TRiP.HM05022), Nach (line TRiP.JF02566) and ppk (line TRiP.JF03250) from TRiP at the Harvard Medical School. The RNAi lines directed to frataxin homolog (fh) have been described (50). All deficiency strains were obtained from the Bloomington stock center, as was UAS-PKCi (51). The PICK1 mutant flies $PICK1^{delEP147}$ and $PICK1^{delEP197}$ (referred to here as delEP147 and delEP197) have been described (26) and were a kind gift from Ole Kjaerulff (The Panum Institute University of Copenhagen). The *rh1-GAL4* driver was a gift of C. Desplan (New York University, New York, USA) and UAS-htau.R406W a gift of M. Feany (Harvard Medical School, Boston, MA, USA).

Histochemistry

Paraffin sections and cryosections were performed as described (47,48). To examine the internal structure of the retina 7 μm paraffin, sections were cut and mounted onto glass slides. Tissue was visualized using the auto-fluorescent property of the fly brain, and are presented in reverse black and white images. For immunohistochemistry, primary antibodies used were mouse anti-HA primary antibody (5B1D10, 1:100, Invitrogen) and mouse anti-Myc (9E10, 1:100, Santa Cruz Biotechnology). Secondary antibodies were anti-mouse conjugated to Alexa Fluor 594 or 488 (1:200 or 1:100, Molecular Probes). Western immunoblots were performed as described (52). Protein was extracted in Laemelli sample buffer (Bio-Rad) from 10 heads of each

genotype, the protein was heat denatured and electrophoresed under denaturing conditions. Each experiment was performed six independent times. Antibodies used were anti-HA antibody conjugated to horseradish peroxidase (3F10, 1:500, Roche), mouse anti-tubulin (E7, 1:2,000, Developmental Studies Hybridoma Bank) and goat anti-mouse IgG (1:2,500, Jackson Laboratories). Signal was detected using Amersham^{TM} ECL Plus and chemiluminescence was detected and quantified on the Fujifilm LAS3000. One-way analysis of variance (ANOVA) followed by a Tukey's test was performed to calculate statistically significant changes in the ratio between soluble SCA3trQ78 protein and tubulin loading control.

Real-time PCR

To determine efficient knockdown of *PICK1*, and *Atx1* by RNAi and for modulation of the *SCA3trQ78* transgene, the relative expression levels were determined by real-time PCR. The trizol method was used to prepare RNA from 10 male flies expressing the *PICK1* RNAi transgene with the *daughterless (da)-GAL4* driver, 20 larvae expressing the *Atx-1* RNAi transgene with the *da*-GAL4 driver and from 10 heads co-expressing *SCA3trQ78* with each of the suppressors under the control of the *rh1-GAL4* driver. Genomic DNA was digested with turbo DNase from Ambion (AM1906). Invitrogen's superscript II was used for first strand synthesis with random priming. For real-time analysis, Applied Biosystems Fast SYBR® Green Master Mix and Applied Biosystems Power SYBR was used. Primers:

PICK1 FP2: 5′ AGCACCCATGTGTCCATGT 3′, *PICK1* RP2: 5′ CTTGCCCTTCACGCTCAC 3′, *β-tubulin* FP: 5′ CATCCAAGCTGGTCAGTG 3′, *β-tubulin* RP: 5′ GCCA TGCTCATCGGAGAT 3′, *Atx-1* FP1: 5′ ATCCAATCGG GAGGCAAC 3′, *Atx-1* RP1: 5′ TCGTCGGAGAACCCATTG 3′, *SCA3* FP1: 5′ CAGGACAGAGTTCACATCCATGT 3′, *SCA3* RP1: 5′ GCCTTACCTAGATCACTCCCAAGT 3′.

Each data set from each sample was normalized to *β*-tubulin, and then each experimental data set was normalized to the control data, all of which were performed using the Applied Biosystems 7500 fast system software. Three technical replicates were performed on three independent samples from each condition. Prism software was used to perform one-way ANOVA followed by a Tukey's test to calculate statistical significance.

Retinal pseudopupil analysis

Ommatidial counts were performed using pseudopupil preparations as previously described (53). Photoreceptor neurons were quantified by counting ommatidia in eyes of female flies d21 highlighted by optical neutralization. Heads of flies were mounted onto glass slides and visualized by a light shining up through the head. Rhabdomeres were counted in 10–20 ommatidia of $n > 10$ female flies of each genotype (100–200 minimum ommatidia/genotype). The mean number of photoreceptors for each ommatidium was plotted from three independent trials. Data are presented as mean ± SEM. The Kruskal–Wallis test was performed using Prism software to calculate statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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