Integration of beta-Catenin, Sirtuin, and FOXO Signaling Protects from Mutant Huntingtin Toxicity

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Neurobiology of Disease

Integration of β-Catenin, Sirtuin, and FOXO Signaling Protects from Mutant Huntingtin Toxicity

J. Alex Parker, Rafael P. Vazquez-Manrique, Cendrine Tourette, Francesca Farina, Nicolas Offner, Arnab Mukhopadhyay, Anne-Marie Orfila, Aurélie Darbois, Sophie Menet, Heidi A. Tissenbaum, and Christian Neri

One of the current challenges of neurodegenerative disease research is to determine whether signaling pathways that are essential to cellular homeostasis might contribute to neuronal survival and modulate the pathogenic process in human disease. In Caenorhabditis elegans, sir-2.1/SIRT1 overexpression protects neurons from the early phases of expanded polyglutamine (polyQ) toxicity, and this protection requires the longevity-promoting factor daf-16/FOXO. Here, we show that this neuroprotective effect also requires the DAF-16/FOXO partner bar-1/β-catenin and putative DAF-16-regulated gene ucp-4, the sole mitochondrial uncoupling protein (UCP) in nematodes. These results fit with a previously proposed mechanism in which the β-catenin FOXO and SIRT1 proteins may together regulate gene expression and cell survival. Knockdown of β-catenin enhanced the vulnerability to cell death of mutant-huntingtin striatal cells derived from the HdhQ111 knock-in mice. In addition, this effect was compensated by SIRT1 overexpression and accompanied by the modulation of neuronal UCP expression levels, further highlighting a cross-talk between β-catenin and SIRT1 in the modulation of mutant polyQ cytotoxicity. Taken together, these results suggest that integration of β-catenin, sirtuin and FOXO signaling protects from the early phases of mutant huntingtin toxicity.

Introduction

Huntington’s disease (HD) is a dominantly inherited CAG repeat disorder with expanded polyglutamine (polyQ) tracts in huntingtin (htt), causing striatal and cortical degeneration (Walker, 2007). While a person carrying more than 39 CAG repeats will invariably develop HD, the age at onset of HD varies considerably. The CAG expansions account for about 60% of the variability of the age at onset of HD, and the residual variability may be heritable, suggesting that other genes influence age at onset (Li et al., 2003; Gayán et al., 2008; Metzger et al., 2008; Taherzadeh-Fard et al., 2009; Xu et al., 2009). Given that HD is an age-related disorder, candidate gene approaches based on longevity modulators have been examined to identify disease modifiers. Studies of TERC1, an evolutionary conserved transcriptional regulator that modulates longevity in Caenorhabditis elegans (Ghazi et al., 2009), showed that this protein is neuroprotective in models of HD (Arango et al., 2006) and that its polymorphic Gln-Ala repeat modifies HD in American patients (Holbert et al., 2001). Another longevity modifier with neuroprotective activities is the transcription factor daf-16/FOXO. FOXO proteins are essential for stress resistance (Brunet et al., 2004), and daf-16 is required for neuroprotection by increased dosage of sirtuin sir-2.1/SIRT1 in expanded-polyQ nematodes (Parker et al., 2005). Several cofactors regulate FOXO activity, and FOXOs have many targets (Greer and Brunet, 2008; Landis and Murphy, 2010; Yen et al., 2011), suggesting that a network of genes centered onto FOXO might regulate mutant polyglutamine neuron survival and have a role in HD variability. Interestingly, in this regard FOXO activity may be conserved from C. elegans to humans, as human FOXO3A was associated with the ability to be long lived in several populations of centenarians (Willcox et al., 2008; Anselmi et al., 2009; Li et al., 2009; Soerensen et al., 2010). Here, we investigated the neuroprotective role of the Wnt effector BAR-1/β-catenin, a protein that also binds to DAF-16/FOXO3a to regulate its transcriptional activity in response to oxidative stress in both C. elegans and mammalian cells (Essers et al., 2005), and that of ucp-4, the
Table 1. Names and genotypes of the *C. elegans* strains used in this study

<table>
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<tr>
<th>Name</th>
<th>Genotype</th>
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*All mutant strains were outcrossed at least three times before use except for extrachromosomal arrays.

*Consortium for Genetics (Center of University of Minnesota, Minneapolis, MN).

*The ID1280 strain contains the transgene from the original strain LG100 and was outcrossed 10 times (see Materials and Methods). It does not contain the dyf mutation, present in the original LG100, and shows no dye-filling defect (see the Results section).

*hst57 refers to amino acids 1-57 of human huntingtin.

*Ht refers to the ucp-4 wild-type promoter. SCR refers to the ucp-4 promoter with the DAF-16 binding motif scrambled (see Materials and Methods).

sole mitochondrial uncoupling protein (UCP) in *C. elegans* and putative DAF-16 transcriptional target. To this end, we used *C. elegans* transgenics expressing exon-1-like htt in touch receptor neurons (Parker et al., 2005). Animals expressing expanded polyQs show a strong neuronal dysfunction phenotype (defective response to touch) in the absence of cell death (Parker et al., 2005), recapitulating an early phase of mutant htt neurotoxicity. Additionally, we tested for the effects of SIRT1, β-catenin, and neuronal UCPS (UCP2, UCP4) on the survival of striatal cells derived from htt knock-in mice (Trettel et al., 2000). As detailed below, our results suggest that integration of β-catenin, sirtuin, and FOXO signaling protects against the early phases of mutant htt toxicity.

### Materials and Methods

**Nematode experiments.** The wild-type strain of *C. elegans* used was Bristol N2. Standard methods of culturing and handling worms, either hermaphrodites or males, were used. All strains were scored at 20°C. Touch tests, scoring of PLM cell processes, drug response assays, and quantitative real-time PCR were performed as described previously (Parker et al.,...
2005). Western analysis was performed using standard protocols and htr fusion proteins detected with the GFP antibody a6556 (Abcam). Mutations and transgenes used in this study are listed in Table 1. All strains were obtained from the C. elegans Genetics Center (University of Minnesota, Minneapolis, MN), except for those generated in the laboratory. For strain construction with polycl transgenes, mutants were verified by visible phenotypes, deletion mutants by PCR analysis, point mutations by sequencing, or a combination thereof. Deletion mutants were outcrossed a minimum of three times to wild type, and the gefs3[sir-2.1(+)] strain was outcrossed 10 times to wild type before use. The gefs3[sir-2.1(+)] animals were tested for dye-filling defect as described previously (Burnet et al., 2011) and showed no defect in this regard.

To test for rescue of the effects of sir-2.1 loss of function (LOF) in 128Q animals, constructs encoding SIR-2.1 were generated as follows. We assembled the sir-2.1 cDNA with a bicistronic GFP (biGFP) by PCR fusion. We obtained the sir-2.1 cDNA from wild-type animals by RT-PCR, using RV197 (5’-GGGGACCACTTTGATACAAAGTTTGTCGTCGCAC-3’) and RV198 (5’-GGGTAAAGGGATGACACGCTATACGGATCAATTCC-3’). Then, we fused both PCR products by nested PCR using primers RV197 and RV178. These primers contain the sequences of attB5 and attB2, respectively, for recombination in the pDONR221-P5-P2 vector using the Gateway system (Invitrogen). In parallel, we produced a clone in pDONR221-P1-P5 containing the promoter of mec-3, mec-3-P5 primer using primers RV3 (5’-GGGGACCATTTGATACAAAGTTTGTCGTCGCAC-3’) and RV178 (5’-GGGGACCACTTTGATACAAAGTTTGTCGTCGCAC-3’). Then, we fused both PCR products by nested PCR using primers RV178 and RV178. These primers contain the sequences of attB5 and attB2, respectively, for recombination in the pDONR221-P5-P2 vector using the Gateway system (Invitrogen). In parallel, we produced a clone in pDONR221-P1-P5 containing the promoter of mec-3, mec-3-P5 primer using primers RV3 (5’-GGGGACCATTTGATACAAAGTTTGTCGTCGCAC-3’) and RV178 (5’-GGGGACCACTTTGATACAAAGTTTGTCGTCGCAC-3’). Then, we fused both PCR products by nested PCR using primers RV178 and RV178. These primers contain the sequences of attB5 and attB2, respectively, for recombination in the pDONR221-P5-P2 vector using the Gateway system (Invitrogen).

To test for rescue of the effects of bar-1 LOF in 128Q animals, constructs encoding BAR-1 were generated as follows. We assembled the bar-1 cDNA with a bicistronic mcCherry (bi-mcCherry) by PCR fusion. We obtained the bar-1 cDNA from wild-type animals by RT-PCR using RV229 (5’-GGTGTCGTCGCAC-3’) and Reverse 5’-CTC-3’ primers. RV230 contains a sequence complementary to the 5’ region of biGFP. We amplified biGFP from pAN51 using RV192 (5’-GGGTAAAGGGATGACACGCTATACGGATCAATTCC-3’) and RV230 (5’-GGTGTCGTCGCAC-3’) primers. We amplified bar-1 from pGEM-MG using primers RV229 (5’-GGTGTCGTCGCAC-3’) and Reverse 5’-CTC-3’. Then, we fused both PCR products by nested PCR using primers RV229 and RV178. These primers contain the sequences of attB5 and attB2, respectively, for recombination in the pDONR221-P5-P2 vector using the Gateway system (Invitrogen). We combined the resulting construct with the plasmid pDONR221-P1-P5 (which contains mec-3P5) to generate the bar-1 overexpression construct by means of the Gateway technology in the destination vector pDEST-AN, which is suitable for C. elegans transgenesis.

Constructs for mcCherry expression under the control of the UCP-4 promoter (1768 bp) were generated as follows. We fused the promoter of UCP-4 (from position 1768 bp upstream of the ATG of ucp-4) and the ucp-4 gene to mCherry:unc-54Terminator. Primers used to amplify ucp-4 were Forward 5’-AAATACATGTTGATCTCTTTTGAATACAGCTGTCGAC-3’ and Reverse 5’-GGGTAAAGGGATGACACGCTATACGGATCAATTCC-3’. Primers used to mutagenize DAF-16 binding site 2 were Forward 5’-AAATACATGTTGATCTCTTTTGAATACAGCTGTCGAC-3’ and Reverse 5’-GGGTAAAGGGATGACACGCTATACGGATCAATTCC-3’. Primers used to mutagenize DAF-16 binding site 2 were Forward 5’-AAATACATGTTGATCTCTTTTGAATACAGCTGTCGAC-3’ and Reverse 5’-GGGTAAAGGGATGACACGCTATACGGATCAATTCC-3’. Primers used to mutagenize DAF-16 binding site 2 were Forward 5’-AAATACATGTTGATC-3’ and Reverse 5’-GGGTAAAGGGATGACACGCTATAC-3’. Then, we fused both PCR products by nested PCR using primers RV178 and RV178. These primers contain the sequences of attB5 and attB2, respectively, for recombination in the pDONR221-P5-P2 vector using the Gateway system (Invitrogen). We combined the resulting construct with the plasmid pDONR221-P1-P5 (which contains mec-3P5) to generate the bar-1 overexpression construct by means of the Gateway technology in the destination vector pDEST-AN, which is suitable for C. elegans transgenesis.
Figure 1. β-Catenin and ucp-4 are required for neuroprotection by sir-2.1 in 128Q nematodes. A, bar-1/β-catenin null mutation enhanced touch insensitivity at the tail of 128Q nematodes. No change was detected in 19Q animals. ***p < 0.001 compared to 128Q alone. B, ucp-4 deletion enhanced 128Q neuronal dysfunction. No change was detected in 19Q animals. ***p < 0.001 compared to 128Q alone. C, 128Q transgene expression is unchanged at the protein and mRNA levels in 128Q nematodes bearing bar-1 or ucp-4 lof. D, Aggravation of neuron dysfunction by sir-2.1 LOF (sir-2.1(ok434)) was suppressed in animals specifically overexpressing (OdE) wild-type SIR-2.1 in touch receptor neurons under the control of the promoter of the mec-3 gene with no effect detected in animals overexpressing empty vector (ID1296 and ID1297; see Table 1). Aggravation of neuron dysfunction by bar-1 LOF (bar-1(ga80)) was suppressed in animals specifically overexpressing wild-type BAR-1 in touch receptor neurons under the control of the promoter of the mec-3 gene, with no effect detected in detected in animals overexpressing empty vector (ID1316 and ID1317; see Table 1). Shown are data compiled from two independent extrachromosomal arrays per genotype. ***p < 0.001 versus empty vector. n.s., Not significant. E, Neuroprotection by increased Sir2 dosage (sir-2.1(OD/E)) against 128Q toxicity was lost in animals mutant for bar-1 or ucp-4. *p < 0.05 versus 128Q; ***p < 0.001 versus sir-2.1(OD/E) alone. Data in A, B, and E are means ± SEM with >200 animals per genotype (50–60 animals per independent experiment for a total of at least 4 independent experiments). Data for touch tests in strains expressing extrachromosomal arrays (D) are means ± SEM with >60 animals per array (20–30 animals per independent experiment for a total of at least 3 independent experiments).

if two different siRNAs showed similar effects on target mRNA/protein expression and cell survival and if scrambled RNAs (25–100 nM) did not show any effect. The active siRNA sequences shown in figures are as follows: hit siRNA, 5'-TTCCGTTACAGCCCGTGATGTT-3' (100 nM); β-catenin siRNA, 5'-GGCTTTTCCCATCCTCATT-3' (100 nM); SIRT1 siRNA, 5'-GATGTGTTAATAATCTCCTT-3' (25 nM); SIRT2 siRNA 5'-TTGGGTGAATGTTCTATTTGTT-3' (25 nM); UCP2 siRNA, 5'-GAAGAAGCAAGCACCTTTA-3' (33 nM); and UCP4 siRNA 5'-CCATTTCCAGGGTATGTT-3' (33 nM). The corresponding scrambled RNAs were as it follows: hit, 5'-GGCTTTTCCCATCCTCATT-3' (100 nM); β-catenin, 5'-GCTTCTGCGCAGTTGATGTT-3' (100 nM); SIRT1, 5'-GTATGCTACATATTAT-3' (25 nM); SIRT2, 5'-GTTGATTTAGTCGCCGTTAGT-3' (25 nM); SIRT3, 5'-GCTATGATACAGCATACTA-3' (25 nM); UCP2, 5'-GCTATGACCTCAGACCATG-3' (33 nM); and UCP4, 5'-GAGCCATTCACTTGACTACTG-3' (33 nM). The construct encoding an unvariant of SIRT1 (2 kb cDNA) lacking an internal segment in the N terminus was pcDNA3.1-sirt1-Flag. Cells were subjected to DAPI staining, and cell death was scored 48 h after cell transfection by counting pyknotic versus normal nuclei in DAPI- and JetS-ENDO-positive cells. Cytoplasmic and nuclear proteins were extracted as described previously (Arango et al., 2006), separated by SDS-PAGE, and analyzed by Western blotting using the following primary antibodies: mouse anti-SIRT1 (Millipore, 1:1000); mouse anti-SIRT2 (Santa Cruz Biotechnology, 1:100), rabbit anti-SIRT-3 (Abgent, 1:100), rabbit anti-β-catenin (Cell Signaling Technology, 1:5000); goat anti-UCP2 (Santa Cruz Biotechnology, dilution), rabbit anti-UCP4 (Abcam, 1:25), mouse anti-htt (4C8, Millipore Bioscience Research Reagents, 1:5000 or GenScript A00089, 1:500), mouse anti-actin (Invtrogen, 1:2000), rabbit anti-NCAM (Millipore Bioscience Research Reagents, 1:10,000). Secondary antibodies were goat anti-mouse IgG HRP-conjugated and goat anti-rabbit IgG HRP-conjugated (Bio-rad). Proteins were detected using ECL+ and evaluated by densitometry using a GS-800 densitometer (Bio-Rad), and quantification was performed using ImageJ. Chemicals were purchased from CalBiochem [6-bromoindirubin-3'-oxime (BIO) and LICE].

RNA Isolation and quantitative RT-PCR in striatal cells. Quantitative RT-PCR was performed on RNA extracted from 109Q/109Q mouse striatal cells transfected with the appropriate siRNAs and scramble RNAs to assess gene silencing efficiency. Two days after cell transfection, RNA was extracted with a Qiagen RNeasy kit and Dnase I (Sigma) treatment (as per the manufacturer’s protocol). Single-strand cDNA synthesis was done using oligo(dT), random hexamer priming, and 100 ng of total RNA with ABSolute 2-Step QRT-PCR SYBR ROX kit (Abgene). Quantitative PCR was performed using SYBR Green with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and oligonucleotides as follows: ucpr-f(5'-TCCGTGACTCTCAGGAAAAC), ucpr-r(5'-TTGGGTGAATGTTCTATTTGTT-3'); ucp2-f(5'-GTACCACTGCTCTTATGCTC-3'), ucp2-r(5'-GAGCCATTCACTTGACTACTG-3'); SIRT3, 5'-CCATTTCCAGGGTATGTT-3' (25 nM); SIRT2, 5'-TTGGGTGAATGTTCTATTTGTT-3' (25 nM); SIRT1, 5'-GTATGCTACATATTAT-3' (25 nM); UCP2, 5'-GCTATGACCTCAGACCATG-3' (33 nM); and UCP4, 5'-GAGCCATTCACTTGACTACTG-3' (33 nM). The construct encoding an unvariant of SIRT1 (2 kb cDNA) lacking an internal segment in the N terminus was pcDNA3.1-sirt1-Flag. Cells were subjected to DAPI staining, and cell death was scored 48 h after cell transfection by counting pyknotic versus normal nuclei in DAPI- and JetS-ENDO-positive cells. Cytoplasmic and nuclear proteins were extracted as described previously (Arango et al., 2006), separated by SDS-PAGE, and analyzed by Western blotting using the following primary antibodies: mouse anti-SIRT1 (Millipore, 1:1000); mouse anti-SIRT2 (Santa Cruz Biotechnology, dilution), rabbit anti-UCP4 (Abcam, 1:25), mouse anti-htt (4C8, Millipore Bioscience Research Reagents, 1:5000 or GenScript A00089, 1:500), mouse anti-actin (Invtrogen, 1:2000), rabbit anti-NCAM (Millipore Bioscience Research Reagents, 1:10,000). Secondary antibodies were goat anti-mouse IgG HRP-conjugated and goat anti-rabbit IgG HRP-conjugated (Bio-rad). Proteins were detected using ECL+ and evaluated by densitometry using a GS-800 densitometer (Bio-Rad), and quantification was performed using ImageJ. Chemicals were purchased from CalBiochem [6-bromoindirubin-3'-oxime (BIO) and LICE].
CATCAGCTAATC-3'). Assays and data analysis were performed according to the manufacturer's protocol (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, PerkinElmer). All samples were run at least in triplicate using rpl13a or hprt as the calibrator gene with a dilution of 1/100 of cDNA. The amount of target, normalized to an endogenous reference (ucp2 or ucp4) and relative to the calibrator (rpl13a or hprt), was calculated using the 2−ΔΔCT method, and statistical significance was determined using paired t tests.

Statistics. Statistics of nematode data were performed using one-way ANOVA, with correction for multiple testing by Tukey's Multiple Comparison Test. Data were expressed as mean ± SEM for >60–200 nematodes in each group, depending on the experiments performed (see figure legends). Student's t tests were used for striatal cell data. Data were expressed as mean ± SD for >150 cells in each group. Student's t test was used for gene and protein expression data. All experiments were repeated at least three times, p < 0.05 was considered significant.

Results
β-Catenin and ucp-4 are required for neuroprotection by increased sir-2.1 dosage in C. elegans

Nematodes expressing normal (19Q) polyQs in touch receptor neurons show a moderate loss of response to touch, whereas nematodes bearing an expanded (128Q) polyQ show a strong loss of response to touch (85%). This touch phenotype is accompanied by axonal swelling and, importantly, occurs in the absence of cell death (Parker et al., 2005), providing an in vivo model to manipulate the early phases of expanded polyQ neurotoxicity.

We previously reported that the sir-2.1/SIRT1-daf-16/FOXO pathway is neuroprotective in expanded polyQ nematodes (Parker et al., 2005). Importantly, we showed that the neuroprotective effects of sir-2.1 overexpression in these animals is unrelated to background mutations that may produce dye-filling defects and influence lifespan in C. elegans (Burnett et al., 2011), and the sir-2.1 overexpression strain used herein does not show dye-filling defects. Recent work in C. elegans has suggested that for DAF-16 to gain specificity in the regulation of lifespan and other biological processes, DAF-16 may interact with different cofactors (Wolff et al., 2006; Landis and Murphy, 2010; Yen et al., 2011). Therefore, to further investigate the regulation of neuronal survival by sir-2.1/SIRT1-daf-16/FOXO, we first examined the evolutionary conserved FOXO cofactor BAR-1/β-catenin. BAR-1/β-catenin directly interacts with both DAF-16 and FOXO3a and has been shown to have a role in oxidative stress protection from diseased neuron vulnerability (Parker et al., 2005), providing an in vivo model to manipulate the early phases of expanded polyQ neurotoxicity.

The UCP-4 promoter is regulated by DAF-16. A, DAF-16 binds to the UCP-4 promoter. The 5′ region of UCP-4 has three consensus DAF-16 binding sites (top; only two sites are shown; binding site 1 has two closely located consensus sites) that are separated by 3.7 kb. One of the binding sites is located 894 bp upstream of the ATG (binding site 2), while two others are located 4,655 and 4,964 kb upstream of ATG. Since the latter sites are closely situated, primers were designed for the site at 4.655 kb (binding site 1). Primers were also designed for region 654 bp downstream of the stop codon as a control site in the 3′-UTR. Chromatin immunoprecipitation (bottom) using anti-DAF-16 antibody (bottom) using N2, daf-2(e1370), daf-16(mgDf50), and daf-16(mgDf50);dafl-2(e1370). The binding was normalized to that of N2, and effects for which p < 0.01 were considered significant. B, Representative images for DAF-16 overexpression to increase the activity of the UCP-4 promoter (1768 bp) in late L4 C. elegans nematodes as inferred from quantifying the intensity of mCherry signals in the pharynx (see Results). C, The effects of DAF-16 on the activity of the ucp-4 promoter requires binding site 2. Constructs encoding mCherry under the control of the ucp-4 promoter (1768 bp), which carried a wild-type (ucp-4 WT) or scrambled (ucp-4 SCR) binding site 2, were stably expressed in N2, daf-16(mu86) or TJ356 strain, and mCherry signals were quantified as indicated in B. Data are mean ± SEM as compiled from two independent arrays (SL1, SL2) per genotype, and >60 animals per array (20–30 animals per independent experiment for a total of at least 3 independent experiments).
signaling (Essers et al., 2005). Loss of function of bar-1 enhanced the loss of response to touch in 128Q nematodes with no effect detected in 19Q nematodes (Fig. 1A) and no change detected in 128Q transgene expression (Fig. 1C), indicating that bar-1 protects from neuron dysfunction induced by expanded polyQs.

Next, we examined a putative DAF-16 target gene, ucp-4, to expand on how daf-16 might affect neuronal survival. The ucp-4 gene encodes for the sole mitochondrial uncoupling protein, UCP, in C. elegans (Iser et al., 2005). We tested ucp-4 for two reasons. First, UCPs might be transcriptional targets of FOXO as suggested by a bioinformatics survey of DAF-16 binding sites in the C. elegans genome (Lee et al., 2003) and a transcriptome analysis of Foxo3a-null mouse neural stem cells (Paik et al., 2009). Second, UCPs are thought to be essential to neuron survival and neurodegenerative disease pathogenesis (Andrews et al., 2009). Neuron dysfunction is aggravated by expanded polyQs in 19Q nematodes (Fig. 1A) and no change detected in 128Q transgene expression (Fig. 1C), indicating that ucp-4 protects from neuron dysfunction induced by expanded polyQs.

In the 128Q nematodes, neuronal dysfunction is intrinsically connected to the dosage of sir-2.1 (Parker et al., 2005; Burnett et al., 2011). Neuron dysfunction is aggravated by sir-2.1 LOF (Parker et al., 2005), and this effect is rescued by overexpression of wild-type sir-2.1 in mechanosensory neurons with no effect of empty vector control, suggesting that this effect of sir-2.1 LOF is cell-autonomous (Fig. 1D). The aggravation of neuron dysfunction by LOF of bar-1 in 128Q nematodes (Fig. 1A) is also cell autonomous, as suggested by the rescue of this effect by overexpression of wild-type bar-1 in mechanosensory neurons with no effect of empty vector control (Fig. 1D). In contrast to aggravation by sir-2.1 LOF, increased sir-2.1 dosage is neuroprotective and requires daf-16 (Parker et al., 2005). Therefore we tested how the DAF-16 cofactor bar-1 and the putative DAF-16 target ucp-4 may also be required for this neuroprotective activity of sir-2.1. The increase in touch response induced by sir-2.1 overexpression was lost in 128Q animals bearing either a bar-1 or a ucp-4 LOF mutation, and the detrimental effects of bar-1 LOF and ucp-4 LOF on touch response were similar in 128Q nematodes and 128Q nematodes bearing increased sir-2.1 dosage (Fig. 1E). Together, these results indicated that the neuroprotective effect of sir-2.1 in addition to requiring daf-16 is also dependent on bar-1 and ucp-4.

The ucp-4 promoter is regulated by DAF-16

Having shown that ucp-4 is required for sir-2.1 to regulate expanded polyQ toxicity in C. elegans neurons, we next tested whether the ucp-4 gene, a putative DAF-16 target (Lee et al., 2003; Paik et al., 2009), may be directly regulated by DAF-16. We scanned the promoter of ucp-4 and identified 3 consensus DAF-16 binding sites within 5 kb upstream of the translation start site (Fig. 2A). We then isolated whole worm extracts from mixed stages populations of wild-type (N2), as well as animals mutant for daf-2/IR/IGF1R or daf-16/FOXO or both, and we performed ChIP with an anti-DAF-16 antibody to test for DAF-16 direct binding to the ucp-4 promoter (Oh et al., 2006; Mukhopadhyay et al., 2008). There was no statistically significant difference in binding signals for the binding site 1 and the 3′ region in daf-16 mutant animals, signifying DAF-16 independent recruitment (Fig. 2A). In contrast, binding signals were strongly decreased for binding site 2 when DAF-16 was absent, signifying DAF-16 dependence (Fig. 2A). To test for the functional role of binding site 2, we generated C. elegans transgenic stocks stably expressing mCherry under the control of the ucp-4 promoter (1768 bp fragment), which carried a wild-type or scrambled DAF-16 binding site 2, and we crossed these strains into a DAF-16 protein overexpression or daf-16 knockout background. First, constructs encoding UCP-4::mCherry under the control of the ucp-4 promoter were stably expressed in a wild-type N2 strain or a strain overexpressing DAF-16::GFP (translational construct TJ356), and the intensity of mCherry signals was quantified in late L4 animals in the pharynx area, an area where DAF-16 isoforms and UCP-4 are known to be expressed (Lee et al., 2001; Lin et al., 2001) using two independent arrays per genotype and >60 animals per array (20–30 animals per independent experiment for a total of at least 3 independent experiments). The expression of mCherry was increased (p < 0.005) in animals bearing DAF-16 overexpression (mean ± SEM: 40 ± 2.7, arbitrary unit) compared to wild-type (mean ± SEM: 57 ± 2.8, arbitrary unit) (see also Fig. 2B). Second, mCherry expression required DAF-16 binding site 2, as shown by the loss of variation of mCherry expression in animals bearing either daf-16 LOF or DAF-16 overexpression when the ucp-4 promoter carried a scrambled sequence for this binding site (Fig. 2C). Together, these experiments suggested that the ucp-4 promoter may be regulated by DAF-16.

Figure 3. The GSK-3β inhibitor BIO is neuroprotective via FOXO signaling in 128Q nematodes. A. Neuron dysfunction in 128Q nematodes is higher compared to 19Q nematodes, with about 85% of the 128Q animals having a defective response to touch (Parker et al., 2005). BIO strongly rescues expanded polyQ neurotoxicity at 100–33.3 μM (p < 0.001 and **p < 0.01 versus DMSO controls) with no effect in 19Q animals. BIO rescuing activity was lost in mutants for daf-16, sir-2.1, bar-1, and ucp-4. Dilution factor is 3×. Percent rescue was calculated from percentages of touch response as (test − control)/(100 − control) × 100. Data are means ± SEM with >200 animals per point for all genotypes (50–60 animals per independent experiment for a total of at least 4 independent experiments). The percentages of touch response (means ± SEM) were 52 ± 3% in 19Q animals treated with vehicle, 23 ± 2% in 128Q animals treated with vehicle, 46 ± 3% in 128Q animals treated with 100 μM BIO, and 34.6 ± 4% in 128Q animals treated with 33.3 μM BIO. B. BIO reduced axonal swelling in PLM cells of 128Q animals (p < 0.002 versus DMSO controls). Data are means ± SEM with >100 animals per treatment (25–30 animals per independent experiment for a total of at least 4 independent experiments). C. BIO treatment (50 μM, 100 μM) does not modify transgene expression in 128Q nematodes. n.s., Not significant.
GSK-3β inhibitors require bar-1, sir-2.1, daf-16, and ucp-4 for neuroprotection

To further expand the mechanistic basis for neuroprotection by sir-2.1 in connection with daf-16 and bar-1 activities, we sought to look at the regulation of β-catenin, a protein that is part of the Wnt signaling pathway and is primarily targeted for degradation by direct phosphorylation by the GSK-3β kinase. Interestingly, previous studies on a simple model system have shown GSK-3β inhibitors such as lithium may protect from expanded polyQs (Berger et al., 2005; Voisine et al., 2007), suggesting that GSK-3β inhibitors may be useful for HD therapy. Therefore, we tested whether sir-2.1, daf-16, bar-1, and ucp-4 may be required for neuroprotection by GSK-3β inhibitors. We first tested BIO, a selective inhibitor of GSK-3β, and observed that at 100–30 µM BIO significantly reduced neuron dysfunction in 128Q animals with no effect detected in 19Q animals (Fig. 3A) and no change detected in 128Q transgene expression (Fig. 3C). However, neuroprotection by BIO was lost in 128Q nematode mutant for any of the four genes including sir-2.1, daf-16, bar-1 and ucp-4 (Fig. 3A), indicating these genes are required for BIO activity. BIO also reduced axonal swelling (Fig. 3B), which may reflect increased neuron health and is consistent with the ability of BIO to reduce the loss of touch response in 128Q nematodes. We also tested lithium chloride, a drug that has GSK-3β inhibition properties, and observed neuroprotective effects similar to those of BIO (data not shown). While BIO and lithium may have secondary targets other than GSK-3β, a genome-scale functional RNAi screen indicated that gsk-3 inactivation is neuroprotective in expanded-polyQ nematodes (Lejeune et al., 2012), suggesting that the neuroprotective effects of BIO and lithium may involve the GSK-3β target. Together, these results suggested that the neuroprotective properties of GSK-3β inhibitors require sir-2.1, daf-16, bar-1, and ucp-4, which may be useful for protecting HD neurons from the dysfunction induced by mutant polyQs.

β-Catenin, SIRT1 overexpression, and UCPs modulate the survival of mouse striatal cells

Having shown that sir-2.1, bar-1, and ucp-4 modulate the neurotoxicity of a N-terminal htt fragment in nematodes, we tested whether their mammalian counterparts may have an effect in a full-length cellular model of HD. To this end, we used striatal cells derived from the htt knock-in mice HdhQ111 (Trettel et al., 2000). Mutant htt (109Q/109Q) striatal cells have a higher rate of cell death induced by serum deprivation compared to wild-type

Figure 4. β-Catenin and UCP2/UCP4 modulate the survival of mutant htt striatal cells from HdhQ111 mice. In these assays, cells were subjected to serum deprivation. A, Representative graph showing that 109Q/109Q cells were more susceptible to cell death compared to 7Q/7Q cells (N = 3 with SD; *p < 0.01 compared to 7Q/7Q). B, Effects of reducing htt levels. Htt siRNA reduced 109Q/109Q cell mortality (N = 3 with SD; **p < 0.01 versus untreated). Scramble RNA showed no effect, ns. Not significant. C, Representative Western blot showing that Htt siRNA treatment reduces Htt expression levels in 7Q/7Q and 109Q/109Q striatal cells. In the following panels, data are normalized due to the variability of striatal cell survival after serum deprivation across experiments. D, Effects of reducing β-catenin levels and of BIO (0.5 µM) treatment. Left, β-Catenin siRNA enhanced 109Q/109Q cell mortality (N = 3 with SD; *p < 0.01 versus untreated). Scramble RNA showed no effect. BIO treatment reduced cell mortality (N = 3 with SD; **p < 0.05 versus DMSO controls). While β-catenin siRNA and BIO were combined, no change in cell mortality was detected compared to cells treated with scramble RNA and DMSO (N = 3 with SD). Right, β-catenin siRNA and BIO showed no effect in 7Q/7Q cells. E, Mutant htt expression was unchanged by treatment with BIO or β-catenin siRNA, β-Catenin levels are increased by BIO (N = 3, p < 0.02) and reduced by β-catenin siRNA (n = 3, p < 0.05). Scramble RNA had no effect. F, Effects of reducing SIRT1, UCP2, and UCP4 (N = 4 with SD). UCP2 siRNA enhanced 109Q/109Q cell mortality (*p < 0.01 versus untreated) with no effect detected in 7Q/7Q cells. UCP4 siRNA reduced 109Q/109Q cell mortality (**p < 0.01 versus untreated) with no effect detected in 7Q/7Q cells. Scramble RNAs had no effect. G, Mutant htt expression was unchanged by siRNAs against SIRT1, UCP2, and UCP4. H, At left is a representative Western blot image showing that 109Q/109Q cells have lower SIRT1 levels upon SIRT1 siRNA treatment (N = 3; p < 0.05). Quantitative RT-PCR experiments indicated that UCP2 and UCP4 siRNA decreased mRNA levels of UCP2 and UCP4, respectively (N = 5 with SD; p < 0.05). The effect of UCP2/4 siRNAs on target protein expression could not be evaluated, as the antisera were repeatedly unable to detect any protein in Western blot experiments, and scramble RNAs showed no effect. For all panels, N indicates the number of independent experiments performed.
(7Q/7Q) cells (Trettel et al., 2000), thus providing an assay to test for cell vulnerability to mutant htt expressed at endogenous levels (Fig. 4A). To test for the implication of htt in cell death induced by serum deprivation, we subjected these cells to htt siRNA treatment using a previously described siRNA (DiFiglia et al., 2007) and corresponding scrambled RNA. Reducing htt levels in 109Q/109Q cells strongly decreased cell mortality upon serum deprivation, with no change in htt expression (Fig. 4C, D). Consistent with the effect of reducing β-catenin levels, the GSK-3β inhibitor BIO enhanced the survival of 109Q/109Q cells (Fig. 4C) with no detectable change in htt expression (Fig. 4D). This amelioration of cell survival was accompanied by increased β-catenin expression (Fig. 4D) and, consistently, was lost when 109Q/109Q cells were incubated with BIO and β-catenin siRNA together (Fig. 4C).

Therefore, in both mouse and C. elegans models for HD, β-catenin has neuroprotective effects, and altering its dosage is detrimental to neuronal cell survival.

Next, we explored the role of SIRTs and UCPs on mouse striatal cell survival. Reducing SIRT1 levels showed no effect on cell survival (Fig. 4E). We tested whether reducing levels of either SIRT2 and SIRT3, two additional sirtuin family members, may modulate striatal cell survival, and observed no effect as well. Therefore, lowering these sirtuins did not affect neuronal survival. Second, we examined the two neuronal UCPs (UCP2, UCP4). UCP2 siRNAs decreased the survival of 109Q/109Q cells (Fig. 4E) with no change in htt expression (Fig. 4F), and they reduced UCP2 mRNA levels (Fig. 4G). In contrast, UCP4 siRNAs increased the survival of 109Q/109Q cells (Fig. 4E) with no change in htt expression (Fig. 4F), and they reduced UCP4 mRNA levels (Fig. 4G), suggesting evolutionary diversity for mouse UCP activity compared to the sole UCP in C. elegans.

Next, we tested whether SIRT1 and β-catenin might cooperate in the modulation of mouse striatal cell survival since sir-2.1/SIRT1 and bar-1/β-catenin are linked to modulate neuron dysfunction in 128Q nematodes. To this end, we overexpressed SIRT1 either alone or in combination with β-catenin siRNA. The overexpression of SIRT1 slightly enhanced the survival of 109Q/109Q cells subjected to serum deprivation with no change in htt levels (Fig. 5A, B). As observed previously, reducing β-catenin levels was strongly detrimental to 109Q/109Q cell survival (Fig. 5A), and this effect was suppressed by SIRT1 overexpression, suggesting that raising SIRT1 levels can compensate for the detrimental effect of β-catenin reduction, which further connected the SIRT1 and β-catenin pathways. We then examined further the individual effects of SIRT1 and β-catenin in mouse striatal cells. Given that UCP gene expression may be regulated by FOXO proteins as suggested by previous studies (Lee et al., 2003; Paik et al., 2009), we tested for the effects of β-catenin reduction and SIRT1 overexpression on the expression levels of UCP2 and UCP4. In mutant htt striatal cells, reducing β-catenin, which aggravates cell death vulnerability, increased mRNA levels of UCP4 (detrimental to cell survival), whereas SIRT1 overexpression, which is slightly neuroprotective, had the opposite effect (Fig. 5C). Conversely, reducing β-catenin decreased mRNA levels of UCP2 (a neuroprotective gene) in mutant htt cells, with however no significant effect of SIRT1 overexpression (Fig. 5C). These results indicated that while SIRT1 and β-catenin may have common
effects such as the repression of UCP4 levels, they may differ in their ability to promote UCP2 levels.

Collectively, these results were consistent with a well-documented model in which the FOXO, SIRT1, and β-catenin proteins may form a complex in the nucleus to regulate gene expression in nematodes and other species (Brunet et al., 2004). Further, these results highlighted a cross-talk between β-catenin and SIRT1 in the regulation of vulnerability to cell death caused by mutant htt expression, which may be primarily mediated by SIRT1 overexpression to compensate for the detrimental effect of reducing β-catenin on cell survival, and by the effects on UCP2 expression levels.

**Discussion**

The Sir2/SIRT1 gene and its target daf-16/FOXO have emerged as potent protective factors for HD and other degenerative diseases (Morley et al., 2002; Parker et al., 2005; Cohen et al., 2006; Kim et al., 2007; Jeong et al., 2011; Jiang et al., 2011). Since FOXO integrates numerous signals and regulates the expression of many genes (Greer and Brunet, 2008; Landis and Murphy, 2010; Yen et al., 2011), it is an important survival factor in normal (Essers et al., 2005) and diseased (Inestrosa and Arenas, 2010) cells through its ability to coactivate either TCF (T-cell factor) or FOXO transcription factors. However, a study of neuronal cell death in HD has suggested that abnormal accumulation of β-catenin may cause neurotoxicity (Godin et al., 2010). Since β-catenin is neuroprotective against the early phases of mutant polyQ cytotoxicity (neuron dysfunction, cell vulnerability), this suggests that β-catenin homeostasis and activity may change along the pathogenic process in HD.

Finally, the activation of cell survival mechanisms controlled by FOXO factors may contribute to delaying HD pathogenesis. Our data indeed suggest that GSK-3β inhibitors require the FOXO network for protecting neurons from the dysfunction induced by expanded polyQs. In addition, neuroprotective effects of SIRT1 activation were recently reported in several mouse models of HD (Jeong et al., 2011; Jiang et al., 2011), and FOXO3a acetylation may be involved in SIRT1 protection (Jiang et al., 2011), which corroborates the C. elegans data. However, we observed differences between C. elegans and mouse striatal cells for UCP activity. This is expected, as evolutionary diversity in gene family function is a widely observed phenomenon. The UCPs can have profound effects on neurons with chronic uncoupling, promoting mitochondrial biogenesis and elevated ATP levels (Andrews et al., 2005b). The enhancement of 12Q toxicity in nematode ucp-4 mutants suggests that ucp-4 normally protect neurons from the dysfunction induced by expanded polyQ expression. However, for mouse striatal cells that express mutant htt, while UCP4 may normally contribute to their vulnerability, UCP2 may protect from it. This is consistent with the neuroprotective effect of UCP2 overexpression in mouse models of Parkinson’s disease (Andrews et al., 2005a). While the C. elegans and mouse striatal cell model both emphasized the neuroprotective effects of β-catenin, they showed differences in their ability to account for the cross-talk between Sir2/SIRT1 and β-catenin. Expanded polyQ nematodes indicated that bar-1/β-catenin is required for sir-2.1/SIRT1 overexpression to be neuroprotective. However, mutant htt striatal cells poorly recapitulated the neuroprotective effects of SIRT1 overexpression and primarily indicated that SIRT1 overexpression compensates for the detrimental effect of β-catenin reduction.

These results highlight the complexity of the regulation around FOXO factors (Greer and Brunet, 2008; Landis and Murphy, 2010; Yen et al., 2011), and they illustrate how the conclusions about the role of cell survival genes may be influenced by the methodological and cellular context in which their activity is assessed. For example, SIRT1 was reported to deacetylate β-catenin and suppress its ability to activate transcription in mammalian cells (Firestein et al., 2008), which appears to contrast with our findings. However, this study was performed in human colon cancer cell lines, where SIRT1/β-catenin homeostasis may be different compared to neurons and cells expressing expanded polyQs. In addition, several studies have indicated that β-catenin is an important survival factor in normal (Essers et al., 2005) and diseased (Inestrosa and Arenas, 2010) cells through its ability to coactivate either TCF (T-cell factor) or FOXO transcription factors. However, a study of neuronal cell death in HD has suggested that abnormal accumulation of β-catenin may cause neurotoxicity (Godin et al., 2010). Since β-catenin is neuroprotective against the early phases of mutant polyQ cytotoxicity (neuron dysfunction, cell vulnerability), this suggests that β-catenin homeostasis and activity may change along the pathogenic process in HD.
might have a role in the modification of the pathogenic process in HD, and this will be addressed in future studies.

References


