SIRT2 inhibition achieves neuroprotection by decreasing sterol biosynthesis

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Huntington’s disease (HD), an incurable neurodegenerative disorder, has a complex pathogenesis including protein aggregation and the dysregulation of neuronal transcription and metabolism. Here, we demonstrate that inhibition of sirtuin 2 (SIRT2) achieves neuroprotection in cellular and invertebrate models of HD. Genetic or pharmacologic inhibition of SIRT2 in a striatal neuron model of HD resulted in gene expression changes including significant down-regulation of RNAs responsible for sterol biosynthesis. Whereas mutant huntingtin fragments increased sterols in neuronal cells, SIRT2 inhibition reduced sterol levels through decreased nuclear trafficking of SREBP-2. Importantly, manipulation of sterol biosynthesis at the transcriptional level mimicked SIRT2 inhibition, demonstrating that the metabolic effects of SIRT2 inhibition are sufficient to diminish mutant huntingtin toxicity. These data identify SIRT2 inhibition as a promising avenue for HD therapy and elucidate a unique mechanism of SIRT2-inhibitor-mediated neuroprotection. Furthermore, the ascertainment of SIRT2’s role in regulating neuronal metabolism demonstrates a central function shared with other sirtuin proteins.

Results

Genetic or Pharmacologic Inhibition of SIRT2 Is Neuroprotective in Models of HD. Given previous evidence that SIRT2 inhibitors ameliorate the neurodegenerative phenotypes of cell and animal models of Parkinson’s disease (12), we asked whether a similar effect could be observed in models of HD. Thus, we first evaluated the recently identified selective and structurally diverse SIRT2 inhibitors AGK2 and AK-1 (12) for their disease-rescuing effects in Drosophila melanogaster expressing N-terminal Htt fragments (N-ter Htt) from human HD exon 1 (Httex1) (5, 13). Freshly eclosed flies expressing Httex1 Q93 in all neurons were fed medium supplemented with AK-1 or AGK2, and neuronal degeneration was assessed 7 days later by using the pseudopupil technique [which scores the number of surviving rhabdomeres (photoreceptor neurons) per ommatidium]. Both inhibitors achieved significant neuroprotection in HD flies at 10 μM (Fig. 1A), improving the number of rhabdomeres from 5.2 to 5.5 and 5.6, respectively. Genetic ablation of SIRT2 also rescued Httex1 Q93-induced photoreceptor neuron death in a dose-dependent manner (Fig. 1B and C; ref. 13).

We next tested whether SIRT2 inhibitors would modulate the neuronal dysfunction associated with expression of N-ter Htt in Caenorhabditis elegans touch receptor neurons (7). Both AGK2 and AK-1 showed significant rescue of mutant polyQ cytotoxicity as measured by improvement in the worms’ defective response to a light touch at the tail (Fig. 1D and E). The improvements of touch response by AGK2 and AK-1 were not attributable to decreased Htt transgene expression (96 ± 12 and 133 ± 85% of controls, respectively).

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SIRT2 inhibition protects against Htt171-82Q toxicity in primary striatal neurons. (A) As shown previously, Htt171-82Q (black bar) exhibits toxicity toward striatal neurons as compared with Htt171-18Q (white bar). AK-1 rescued Htt171-82Q-expressing cells in a dose-dependent manner (at concentrations of 1, 2, and 4 µM) (A) and significantly reduced the number of mutant Htt positive inclusions (B). AGK2 also rescued striatal neurons from Htt171-82Q toxicity (C) and significantly reduced the number of inclusions (D). Overexpression of SIRT2WT abrogates neuroprotection by AK-1 (E). A lentiviral vector encoding CFP is used as a co-infection control. A dominant negative deacetylase mutant SIRT2H150Y significantly decreases Htt171-82Q toxicity in primary striatal neurons (F) and also significantly reduced the number of inclusions (G). A lentiviral vector encoding CFP is used as a co-infection control (* P < 0.05).
inhibition accounts for the observed AK-1- and AGK2-mediated neuroprotection.

**SIRT2 Is Not Overexpressed in HD.** Because distinct changes in RNA and protein expression have been associated with mutant Htt toxicity (3), we next explored whether a pathological increase in SIRT2 might be the disease-related effect targeted by SIRT2 inhibition. Because previous descriptions of the expression of SIRT2 in the mammalian brain showed it to be localized primarily to oligodendrocytes (11, 16), we first verified SIRT2 expression in neurons, where mutant Htt’s effects are greatest. Mouse brain sections showed extensive colocalization of SIRT2 and CNPase (2′,3′-cyclic nucleotide 3′-phosphodiesterase) immunoreactivities, including in white matter bundles within the striatum, in concordance with the reported localization of SIRT2 in oligodendrocytes (Fig. S3 A), but we also observed colocalization of SIRT2 immunoreactivity with the neuronal marker NeuN in cortical and striatal neurons (Fig. S3 B and C). Furthermore, Western blots also detected ≈43 kDa anti-SIRT2-immunoreactive bands in neuron-like cell lines, mouse cerebral cortex, and rat primary neurons (Fig. S3 D–F). However, equivalent expression and localization of SIRT2-immunoreactive species were observed in both normal and HD model conditions [wild-type and transgenic HD (R6/2) mice and Htt171-18Q- and Htt171-82Q-expressing striatal neurons] (Fig. 3 B–D). Thus, we concluded that SIRT2 is present in cortical and striatal neurons but is not up-regulated in HD.

### Inhibition of SIRT2 Down-Regulates Genes Involved in Sterol Biosynthesis.

**The known histone deacetylase activity (17), nucleo-cytoplasmic shuttling (9, 18, 19), and experimentally verified neuronal expression of SIRT2 (see above) led us to hypothesize that SIRT2 might regulate neuronal transcription; we therefore postulated that modulation of neuronal gene expression might be the cellular mechanism of SIRT2 inhibitor-mediated neuroprotection. Mutant Htt is known to wield major effects on steady-state mRNA levels through either direct or indirect transcriptional regulation (20). Primary neurons expressing mutant Htt fragments faithfully recapitulate HD-related changes in RNA expression (21) and, thus, provide a suitable model in which to evaluate modulatory effects of gene expression by SIRT2 inhibition. As expected, Htt171-82Q expression in striatal neurons resulted in significant gene expression effects as compared with Htt171-18Q-expressing cells (116 decreases and 36 increases by criteria of calyceal uncorrected by SIRT2 inhibitor treatment (Fig. S4 and Dataset S1).

We next assessed whether other gene regulatory effects might account for SIRT2-mediated neuroprotection. Interestingly, short-term treatment with SIRT2 inhibitor AK-1 produced large statistically significant changes in RNA expression in untransduced, Htt171-18Q- and Htt171-82Q-expressing neurons. To define the functional effects of SIRT2-related gene regulation, we assessed which biological pathways were represented by genes responding to SIRT2 inhibition. Strikingly, molecular pathway analysis of SIRT2-regulated genes according to Gene Ontology classification showed highly significant overrepresentation of metabolic cascades, the most prominent of these being decreased expression of genes associated with sterol biosynthesis (Fig. 3 A and Dataset S2). Although these findings are consistent with previous data implicating other sirtuins in cellular metabolism (8), they are surprising and unique with respect to the previously known roles of SIRT2. These data raised the intriguing possibility that the cellular neuroprotective mechanism of SIRT2 inhibition might be the negative regulation of sterol production. We confirmed the microarray results in new samples of striatal neurons treated with AK-1 and AGK2; these assays reproduced the down-regulation of cholesterol biosynthetic genes (Fig. S5 A and C) and also showed a significant decrease in the levels of sterols (cholesterol and cholesterol esters; see Materials and Methods) (Fig. S5 B and C). These data demonstrate that AK-1- and AGK2-mediated neuroprotection is correlated with the negative regulation of sterol biosynthesis.

We next asked whether the metabolic regulation mediated by AK-1 and AGK2 was specifically attributable to their inhibition of SIRT2. We therefore assessed whether inhibition of SIRT2 via the overexpression of the deacetylase-deficient SIRT2H150Y mutant would result in the same down-regulation of sterol biosynthesis genes and sterol levels. Although overexpression of wild-type SIRT2 had no effect on sterol biosynthesis, SIRT2H150Y significantly decreased sterol levels and RNAs encoding cholesterol biosynthetic enzymes (Fig. S5 E and F). The fact that genetic manipulation of SIRT2 activity reproduces the gene regulatory effects of SIRT2-targeting small molecules substantiates the on-target effects of these inhibitors. Moreover, these data convincingly demonstrate a role of SIRT2 in regulating cellular metabolism.

**SIRT2 Inhibition Reduces polyQ-Induced Cholesterol Dyshomeostasis.** The correlation of decreased sterol biosynthesis and neuroprotection in our experiments raised the possibility that the negative regulation of sterol production by SIRT2 inhibitors...
directly opposed a toxic dysregulation of sterol homeostasis by Htt171-82Q. Moreover, previous literature has also described effects of mutant Htt on sterol-related pathways (22, 23). Therefore, we examined the effects of mutant Htt fragments on sterol content in our primary striatal neuron model of HD. Indeed, Htt171-82Q expression increased cellular sterol levels (cholesterol and cholesteryl esters; see Materials and Methods) compared to CFP or Htt171-18Q controls (Fig. 3B). Consistent with the previous results, AK-1, AGK2, and SIRT2150Y were able to diminish sterol species in Htt171-82Q-expressing neurons (Fig. 3B–D). These results support the model that polyQ-induced sterol dyshomeostasis contributes to HD-related neurotoxicity, whereas inhibition of SIRT2 down-regulates sterol biosynthesis and abates this toxicity.

**SIRT2-Mediated Effects Are Driven by Extranuclear Events.** Our immunochemical studies (Figs. S2A and S3A–C) had shown that neuronal SIRT2 was localized to both nuclear and extranuclear compartments. Based on the gene regulatory effects of SIRT2 inhibition, we postulated that the observed neuroprotective and gene regulatory effects involved direct nuclear actions. We therefore overexpressed SIRT2 variants with nuclear import and export mutations to explore this question. Counter to our prediction, however, nuclear targeting of SIRT2 negated its ability to block neurodegeneration by AK-1, whereas extranuclear targeting efficiently antagonized AK-1’s effect (Fig. S6A and B). Likewise, cellular sterol levels were increased by extranuclear compared with nucleus-targeted SIRT2 (Fig. S6C). These results show that both the neuroprotective and metabolic effects of SIRT2 inhibition are mediated by cellular events that occur in extranuclear compartment(s).

**SIRT2 Regulates the Nuclear Trafficking of SREBP-2.** We next investigated the mechanism by which SIRT2 might regulate sterol biosynthesis. Visualization of gene networks with Ingenuity Pathway Analysis showed that many of the genes down-regulated by AK-1 were all controlled by a common transcription factor: the sterol response element binding protein 2 (SREBP-2); we therefore hypothesized that SREBP-2 might be an important mediator of SIRT2-related neuroprotection. SREBP-2 activity is known to be controlled by its necessary translocation from the endoplasmic reticulum to the nucleus, where it can bind to SRE enhancer elements in DNA and increase cholesterol biosynthesis. Thus, we tested whether SIRT2 activity regulated the nuclear trafficking of SREBP-2. Indeed, chemical inhibition of SIRT2 with AK-1 or AGK2 led to a reduction in the nuclear compartmentalization of SREBP-2 (Fig. 4A). Furthermore, overexpression of wild-type SIRT2 (or extranuclear SIRT2) increased the percentage of nuclear SREBP-2 labeling in primary neurons, whereas SIRT2150Y (or nuclear SIRT2) had the opposite effect (Fig. 4B and Fig. S7). Combined with the observed down-regulation of a large set of known SREBP-2 target genes and decreased cellular sterol levels, these results identified SREBP-2 as a crucial mediator of the effects of SIRT2 on metabolism.

**Metabolic Regulation via SREBP-2 Inhibition Conveys Neuroprotection.** Although the above results show that down-regulation of sterol biosynthesis correlates with SIRT2-inhibitor-mediated neuroprotection, they do not establish causality. Therefore, the remaining question to be addressed was whether the negative regulation of SREBP-2 by SIRT2 inhibition was sufficient to achieve neuroprotection. We tested this hypothesis by expressing dominant-negative (SREBP-2NEG) versus constitutively active SREBP-2 (SREBP-2ACT) mutants in cultured neurons (Fig. S8A–D) and assessed their effects on polyQ toxicity. Consistent with our hypothesis, the dominant-negative SREBP-2 conveyed significant neuroprotection to Htt171-82Q cells (Fig. 4C) but had no effect on the number of mutant Htt-positive inclusions (Fig. S8E). Conversely, the constitutively active SREBP-2 enhanced polyQ toxicity (Fig. 4D). Moreover, the neuroprotective effect of AK-1, AGK2, and SIRT2150Y was circumvented by addition of constitutively active SREBP-2, which is not targeted to the endoplasmic reticulum (Fig. S8F–H). These data demonstrate that the regulation of metabolism is an important mechanism of the neuroprotective activity conveyed by SIRT2 inhibitors.

**Discussion**

**SIRT2 Demonstrates Neuroprotection in Models of HD.** Previously we demonstrated that SIRT2 inhibition prevented neurodegeneration in models of Parkinson’s disease (12). Here, we further validate that our chemical SIRT2 inhibitors mediate neuroprotection through on-target activities and extend the potential applications for such inhibitors to Huntington’s disease, another fatal neurodegenerative disorder. We also report a breakthrough discovery regarding the metabolism-related mechanism(s) through which SIRT2 inhibition acts by elucidation of its gene regulatory effects. SIRT2 inhibition by either genetic or chemical means resulted in a decrease in Htt inclusion accumulation and increased neuronal viability. The recapitulation of SIRT2 inhibitor-mediated neuroprotection through negative regulation of sterol biosynthesis and the blockade of neuroprotection by circumventing this regulation demonstrate that this metabolic effect contributes significantly to Htt pathology. Previous studies have also assessed mutant Htt’s effects on sterol homeostasis. These analyses reported the accumulation of cellular cholesterol attributed to the binding of mutant Htt to caveolae (23), altered levels of cholesterol biosynthesis-related RNAs and sterols, including decreased 24-OH-cholesterol.
(22, 24, 26, 27), and a direct (albeit weak) interaction with SREBP-2 (28). The evidence available to date does not yet provide a complete understanding of mutant Htt’s effects on sterol homeostasis, however. Although differences in assay measures may account for some heterogeneity in findings, we believe that a more comprehensive explanation resides in the fact that the mechanisms of brain sterol regulation, distinct contributions of different cell types, and varying effects depending on nutrient status still remain to be elucidated. Our working hypothesis is that a plurality of sterol-pathway-related mitigatory effects exists and that the balance of these activities and specific nutrient conditions determines the net outcome. This perspective is consistent with recent findings by Valenza and Cattaneo demonstrating complex changes in sterol biosynthesis and compensatory homeostasis in the brains of R6/2 mice (24). Given the generally accepted importance of sterol regulation to brain function, it seems likely that a more detailed understanding of the mechanisms of sterol-related neuroprotection will allow important insights for HD therapy.

**Unique Role of SIRT2 in the Regulation of Neuronal Cholesterol.** Here we provide strong evidence of a unique and important regulatory function of the SIRT2 deacytase in neuronal cells. Biological pathway analysis indicated that prominent effects of SIRT2 comprise the regulation of genes controlling metabolism, including sterol and fatty acid biosynthesis, carbohydrate metabolism, and purine metabolism (Dataset S2). An apprarent role for SIRT2 in regulating lipid and sterol biogenesis is consistent with its previously reported localization in oligodendrocytic myelin sheaths (16) and role in adipocyte differentiation (29). Conversely, however, no previous studies implicate SIRT2 in the regulation of cholesterol biosynthesis and, thus, the findings of this study motivate further examination of SIRT2 in this role.

Studies of late have shown detrimental effects of cholesterol accumulation in neurons and substantiate the potential benefit of decreasing neuronal cholesterol (or other sterol species) as a neuroprotective strategy. Although there has been amiable evidence in the more distant past, a flurry of recent reports have clarified the detrimental effects of cholesterol accumulation in neurons (30–32). These studies are paralleled by evidence that increased neuronal cholesterol presents a risk factor for Alzheimer’s and Parkinson’s diseases (33–35). Although debated, it has also been proposed that neuronal cholesterol may have an intrinsic biosynthesis (36), rather than transport from extracellular sources. Moreover, other aspects of the regulation of neuronal cholesterol homeostasis are also unique, such as the important role of 24-OH-cholesterol and its biosynthesis by CYP46A1. In fact, CYP46A1 has been deemed a master regulator of neuronal cholesterol biosynthesis based on the evidence that 24-OH-cholesterol has a more significant role than the SREBP cleavage-activating proteins (SCAPs 1 and 2) in regulating the nuclear translocation of SREBP-2 in this cell type (37, 38).

**Molecular Mechanisms of SIRT2-Related Metabolic and Neuroprotective Activities.** Although we have established that SIRT2 modulation controls the nuclear trafficking and transcriptional activity of SREBP-2, the specific molecular determinants of this regulation remain to be elucidated. A priori, the assumption would be that the deacytlation of a SIRT2 substrate (or substrates) is responsible for this effect. It is well established that α-tubulin is a substrate of both SIRT2 and its cytoplasmic interacting protein HDAC6 and, thus, inhibition of SIRT2–HDAC6 complexes might affect microtubule-dependent trafficking. Therefore, it is plausible to propose α-tubulin as the effector whose increased acetylation inhibits SREBP-2 translocation. Alternatively, other proteins, although not previously named as SIRT2 substrates, would also comprise obvious candidate effectors because of their known roles in the regulation of neuronal cholesterol homeostasis. These candidates include SREBP-2 itself or the proteins regulating the sequestration of SREBP-2 in the endoplasmic reticulum, namely the SCAPs, the site 1 and 2 proteases, and CYP46A1. Intriguingly, we note that both SREBP-2 and CYP46A1 contain lysines with high context prediction scores for acetylation (obtained using Prediction of Acetylation on Internal Lysines [PAIL] [http://bdmpail.biocuckoo.org/prediction.php]).

Although we have provided evidence for the contribution of decreased SREBP-2 activity in SIRT2 inhibitor-mediated neuroprotection, we have not ruled out parallel contributions of other SIRT2-dependent effects. For example, increasing α-tubulin acetylation could ameliorate HD pathogenesis by independent mechanisms such as regulating mutant Htt aggregation or degradation (39) or the transport of membranous vesicles, including those containing BDNF (40). In fact, our observation that both genetic and pharmacologic inhibition of SIRT2 leads to reduced numbers of mutant Htt inclusions, whereas inhibition of SREBP-2, although protective, does not alter inclusion numbers, further supports the perspective that other SIRT2-dependent processes may have independent mitigating effects.

**Role of SIRT2 in the Regulation of Metabolism Draws Parallels to Other Sirtuin Activities.** The newly identified role of SIRT2 as a regulator of metabolism in neurons establishes a previously undisclosed function shared with other members of the sirt2/SIRT family (8). Roles for sirt2 and SIRTs 1, 3, 4, 5, and 6 in the control of cellular metabolism have been well established in lower organisms and in nonneural mammalian tissues, respectively (8). Previous data as well as those of the present study suggest that sirtuin proteins comprise a complex network of metabolic regulators whose activities are present in a number of molecular pathways in a variety of tissues. Although we have provided substantial evidence for SIRT2’s role in cholesterol biosynthesis, the other potential metabolic activities suggested by the pharmacogenomic profile of AK-1 remain to be determined.

**Implications for Other Age-Related Diseases.** Whereas increased activity of cholesterol biosynthetic enzymes and cholesterol accumulation generally occurs during aging, lower cholesterol levels have been associated with peripheral and central benefits, including increased lifespan and decreased amyloid accumulation (41, 42). Because the brain is previously known to be less sensitive to dietary modulation than peripheral tissues (36), it is particularly interesting to consider unique ways to regulate brain cholesterol. As shown in the present study, this regulation might be achieved through SIRT2 inhibition, barring the remaining challenges of developing brain-permeable small-molecule SIRT2 inhibitor compounds and assessing potential complications of SIRT2 inhibition in nonneuronal cells (most notably, oligodendrocytes). However, if SIRT2 also regulates cholesterol synthesis beyond the nervous system, then even non-brain-permeable SIRT2 inhibitors may be considered for the management of peripheral disorders involving hypercholesterolemia or vascular disease. Thus, the unique facets of SIRT2 activity identified in this report warrant further study in a broader health-related context.

**Materials and Methods**

Detailed methods appear in the **SI Materials and Methods**. These include Drosophila feeding and omni assay analysis, drug evaluation in C. elegans, antibodies and reagents, plasmids, lentiviral vectors, primary cultures, immunocytochemistry, inclusion analysis, Western blot, mouse extract preparation, immunohistochemistry, gene expression profiling, cholesterol assay, evaluation of SREBP-2 compartmentalization and statistical analysis.

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Supporting Information

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SI Materials and Methods

**Drosophila Feeding and Ommatidial Analysis.** UAS-Httex1p Q93 (line P463) flies were mated to elav > Gal4 at 25 °C, and the freshly eclosed poly-O-expressing females transferred into vials containing standard *Drosophila* medium supplemented with either AGK2, AK-1, or vehicle (DMSO). Flies were transferred to fresh food every day and assayed for neurodegeneration at 7 days posteclosion by using the pseudopupil technique (1). At least 30 ommatidia in each of the 4–8 flies were scored, and the average number of rhabdomeres per ommatidium was calculated for each fly. Two independent trials were conducted. Significance of the difference from the no compound (DMSO) control was evaluated by using a Student’s *t* test.

For SIRT2 mutants, crosses were done as follows: elav; Sirt2[5B-2-35]SB males were crossed to Httex1p Q93 (P463) or Httex1p Q93 (line P463) females (a recombinant line made from our Htt line and the Sirt2 mutant). The flies were raised at 25 °C and assayed 7 days after eclosion. The [5B-2-35] allele is a null generated by K.G. Golic (University of Utah, Salt Lake City): It is a deletion of the Sirt2 coding region produced by ends-in gene targeting followed by CreI-Cre1-induced deletion.

**Drug Evaluation in C. elegans.** Touch tests and drug response assays were performed as described (2, 3).

**Mouse Extract Preparation for Western Blotting.** Extracts of wild-type and R6/2 right frontal cortex were prepared from acutely dissected tissues. Each sample was weighed and homogenized in 20 volumes of 1× SIRT buffer (50 mM Tris-Cl at pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mg/mL BSA) + 10% glycerol. Fifty-microliter fractions of total lysate were removed, and remaining lysates were centrifuged at 18,000 × *g* for 10 min. Each supernatant and pellet fractions were collected. The pellet fractions were resuspended in 1× SDS sample buffer + DTT (in 10% of the original sample volume), boiled at 100 °C for 5 min, and sonicated two times for 2 min each. Five microliters each of pellet and supernatant fractions were used for Western blotting.

**Immunohistochemistry and Confocal Microscopy of Mouse Brain.** Whole brains were frozen in isopentane on dry ice and stored at −80 °C. Sections were cut to 15 μm by using a cryostat (Brights Instruments) and mounted onto glass slides. Sections were postfixed in 4% paraformaldehyde for 30 min or 100% methanol at −20 °C for 10 min, and then washed with PBS for 15 min. Nonspecific sites were blocked by incubation in PBS containing 2% BSA. Sections were incubated in primary antibody diluted in blocking solution overnight at 4 °C, washed in PBS and incubated for 1 h at RT in fluorescent secondary antibodies and nuclear stain (TO-PRO-3) in 1:1,000; Molecular Probes) in blocking solution. After washing in PBS the sections were mounted in Mowial media. Sections were visualized by using an LSM 510 confocal microscope (Zeiss).

**Antibodies and Reagents.** Primary antibodies and relevant concentrations include the following: mouse monoclonal anti-Neuronal Nuclei (NeuN) (Chemicon MAB377 clone A60; ICC–1:400, IHC–1:200), mouse monoclonal anti-acyetylated tubulin (Sigma T7451 clone 6–11B-1; WB: 1:10,000), mouse monoclonal anti-GAPDH (Abcam Ab8245 clone 6C5; WB–1:30,000), mouse monoclonal anti-c-myc (EPFL in-house clone 9E10; ICC–1:20,000), mouse monoclonal anti-HA (Covance MMS-101R clone 16B12; ICC–1:500), mouse monoclonal anti-CNPase (Abcam ab6319; IHC–1:1,000), rabbit polyclonal anti-SIRT2 (Santa Cruz H-95 sc-20966; IHC–1:100/200), rabbit polyclonal anti-SIRT2 (Sigma S8447; ICC–1:200, WB–1:2000), rabbit polyclonal anti-SREBP-2 (Abcam ab30682; ICC: 1:200), mouse anti-huntingtin (Chemicon MAB5492 clone 2B4; ICC–1:50). Secondary antibodies and relevant concentrations include the following: donkey anti-mouse IRDye 800CW (LI-COR Biosciences 926–32212; WB–1:20,000), donkey anti-rabbit IRDye 680 (LI-COR Biosciences 926–32223 WB–1:20,000), goat anti-mouse IgG (H+L) Cy3 552 (Jackson ImmunoResearch Laboratories 115–166–003; ICC; 1:1000), donkey anti-mouse IgG (H+L) Alexa Fluor 550 (Invitrogen Molecular Probes A11057; IHC–1:1000), donkey anti-mouse IgG (H+L) Alexa Fluor 488 (Invitrogen Molecular Probes A11008; IHC–1:1000), goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Invitrogen Molecular Probes A11008; ICC–1:500), goat anti-mouse IgG (H+L) Alexa Fluor 594 (Invitrogen Molecular Probes A11052; IHC–1:500). All treatments of primary neurons with AK-1 and AGK2 (Cambridge) were at 10 μM and 7.5 μM respectively for 24 h unless otherwise indicated. Experiments with lower doses were chronically treated once per week beginning at DIV 4, coinciding with normal medium change and continuing until the experimental endpoint. Equivalent concentrations of dimethyl sulfoxide vehicle served as control.

**Plasmids.** Total RNA was harvested from fresh rat brain homogenate, and reverse transcriptase-PCR was used to obtain cDNA for genes of interest. The cDNAs encoding the rat SIRT2 (SIRT2WT) (NM_001008368; nucleotides 467–1519), human SREBP-2 (NM_001033694; nucleotides 141–1514 encoding amino acids 1–458 SREBP-2ACT and 1023–1514 encoding amino acids 295–498 SREBP-2N5E), CFP, and YFP were inserted downstream from the mouse phosphoglycerate kinase 1 (PGK) promoter in a self-inactivating lentiviral transfer vector (SIN-W-PGK). A sequence encoding a c-myc epitope (EQKLISEEDL) was cloned into the C terminus of all SIRT2 forms and an HA epitope (YPYDVPDYA) into the N terminus of SREBP-2 clones. A Kozak consensus sequence (CCACC) was inserted upstream from the ATG. Site-directed mutagenesis using extension overlap sub-stituted a thymidine for cytosine at nucleotide 914, which led to the histidine substitution for tyrosine at position 150. SIRT2N5E was created by the addition of a sequence encoding the nuclear export signal from HIV Rev (LPLLERLTL) to the C terminus of SIRT2. SIRT2N5ES+NLS was created by deletion of an endogenous N-terminal nuclear export signal (amino acids 4–14 encoding LRNLFTQTLGL) (4) and addition of the nuclear localization signal from the SV40 large T antigen (PKKKRKV) to the C-terminal end. Plasmids encoding huntingtin were comprised of the first 171 amino acids of human huntingtin containing either 18 or 82 CAG repeats under control of a TRE-regulated promoter (SIN-TRE-Htt171-18Q/82Q-WPRE). Expression was stimulated with the tTA transactivator (SIN-PGK-tTA-WPRE).

**Lentiviral Vectors.** Lentiviral vectors were produced in human embryonic kidney 293T (HEK293T) cells with a four-plasmid system as described (5). The viruses were resuspended in PBS with 1% of BSA and matched for particle content to 1,500 ng of p24 antigen per milliliter (1 ng of p24 per 10,000 cells) as measured by ELISA (RETROtek; Gentaur). A p24 of 750 ng/mL was used for SIRT2H405Y neuroprotection analysis and SIRT2WT for blocking the AK-1 protective effect. Viral stocks were stored at −80 °C until use. Cultures were infected with huntingtin-encoding viruses.
1 day after plating, and neuroprotection was assessed after 3 weeks. All other viruses were infected on DIV 4.

**Primary Cultures.** Dissociated neuronal cultures were prepared from ganglionic eminences of embryonic day 16 rat embryos, yielding a majority population of neuronal nuclear antigen (NeuN)-positive neurons and some residual astroglial cells (5). HEK293T cells were used for dominant negative testing of SIRT2H150Y and were transfected by using a CaPO4 procedure.

**Gene Expression Profiling.** Samples comprised of primary striatal neuron cultures were treated for 24 h with vehicle (0.1% DMSO), 10 μM AGK2, or 10 μM AK-1 (n = 5 independent culture wells for each treatment). Samples assessing effects in polyglutamine protein-expressing cells were infected with lentiviral vectors expressing Htt171-18Q (control Htt fragment) or Htt171-82Q (mutant Htt fragment), as per Zali et al. (5), and analyzed after 6 weeks in vitro. RNA was extracted by using the RNeasy system (Qiagen), following the manufacturer’s protocol. For uninfected samples, 1 μg of total RNA was used to prepare biotinylated fragmented cRNA, which was prepared, hybridized to Rat Genome 230 2.0 microarrays, washed, stained, and scanned according to the GeneChip Expression Analysis Protocol employing products from Affymetrix. Samples from Htt171-expressing cultures comprised 100 ng of total RNA, which were amplified by using the WT-Ovation FFPE RNA Amplification System. Samples were fragmented and labeled by using the Ovation Biotin RNA Amplification and Labeling System, and 6 μg of the resultant cDNA were hybridized to GeneChip Rat Genome 230 2.0 Arrays. For all samples, gene expression was quantified by robust multiarray analysis (6) using the R software package affy (7). All statistical analyses of gene expression were carried out with the R software package limma (8) by using a False-Discovery Rate (FDR) approach to correct for multiple testing (9), with a significance threshold of FDR P < 0.05. Drug effects on gene expression were assessed in comparison with vehicle-treated samples; overlapping drug effects changes were defined by sign and FDR threshold of differential expression for each probeset. Reported results use Affymetrix annotation dated 07/12/2007 (Rat230_2.0a23.annot.csv). Over- and underrepresentation of Gene Ontology (GO) terms (in the “biological process” branch) among significant gene expression changes detected after AK-1 treatment was performed by using the software GOstat (10), choosing “Yekutieli” as the multiple testing correction approach. Additional exploratory analyses of biological pathways were conducted using Ingenuity Pathways Analysis (www.ingenuity.com). Quantitative real-time PCR (QPCR) analysis used the same protocols for RNA preparation, and samples were analyzed by using Applied Biosystems 7900HT Real-Time PCR System and SDS 2.5 software. Sample cDNA input ranged from 1 to 2.5 ng per test.

**Immunocytochemistry.** Cells were fixed for 10 min in 3% paraformaldehyde (Sigma)/PBS, then permeabilized in 0.5% Nonidet P-40 (Sigma)/PBS for 1 min, followed by an additional 2 min in paraformaldehyde and blocked in 3% BSA (Sigma)/PBS for 30 min. Primary antibodies, diluted in BSA/PBS, were applied for 30 min followed by three rinses in PBS, then exposed to an Alexa-conjugated secondary antibody (Invitrogen Molecular Probes), diluted 1:500, for 30 min, and finally rinsed in PBS. NeuN immunocytochemistry was performed identically except 0.1% Triton X was included in blocking and antibody solutions in place of Nonidet P-40 permeabilization, normal goat serum (Dako-Cytomation) was used for blocking, primary antibody was incubated overnight at 4 °C, and a Cy3 (Jackson Immunoresearch Laboratories) secondary antibody was used. Images for quantitative analysis were acquired with a BD Pathway 855 Biomager (BD Biosciences) under nonsaturating exposure conditions and analyzed with NIH Image J software. Confocal images were acquired with a Leica SP2 Upright by using LCS 2 software.

**Inclusion Body Analyses.** Five biological replicates (≈50,000 plated cells) of primary neurons expressing mutant Htt fragments and treated with DMSO, AK-1, or AGK2, or coinfected with CFP or SIRT2H150Y, were labeled with anti-huntingtin (clone 2B4) as described above. Images were obtained with the BD Pathway 855 Biomager, and analysis was performed by using ImageJ software with varying size thresholds.

**Western Blot Analyses of Primary Cultures.** Cells were harvested in 1% Triton X buffer containing 20 mM Hepes at pH 7.4, 2 mM EDTA, 2 mM EGTA, 100 mM KCl, 1× protease inhibitor mixture, and 2 mM phenylmethylsulfonfonyl fluoride. All chemicals were purchased from Sigma. Samples were separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked for 1 h in 30% Odyssey Blocking Buffer (LI-COR Biosciences)/PBS containing 0.1% Tween-20 (Sigma) followed by primary antibody diluted in blocking solution overnight at 4 °C. Membranes were rinsed with 0.1% Tween-20/PBS then secondary antibody diluted in blocking solution was applied for 2 h at room temperature, followed by rinsing and scanning with a LI-COR Odyssey Infrared Imager (LI-COR Biosciences).

**Cholesterol Assays.** Neurons were rinsed with 1× PBS and harvested in buffer (Invitrogon) containing 0.1 M potassium phosphate at pH 7.4, 50 mM NaCl, 5 mM choline acid, and 0.1% Triton X-100. Samples were frozen and thawed before raw cholesterol values were obtained with a Tacon GENios Pro (Tecan) by using the Amplex Red Cholesterol Assay Kit (Invitrogen Molecular Probes) according to manufacturer’s instructions. This procedure measures 3β-hydroxysteroids and 3β-hydroxysterol esters, which are substrates of cholesterol oxidase and cholesterol esterase, respectively. Cholesterol comprises ~91% of free sterol in rat brain, whereas cholesteryl and methylocholesteryl esters, respectively, comprise 54% and 46% of sterol esters (11). Sterol values were normalized to total protein as assessed with the BCA Protein Assay (Pierce).

**Evaluation of SREBP-2 Compartmentalization.** Images of 30 random fields from blinded conditions were taken by using a Leica DMI 4000 microscope (×40 magnification) and blindly evaluated by using Image J to evaluate the pixel density in traced NeuN positive nuclei as a percentage of the pixel density of the entire cell body. The number of neuronals evaluated for each condition were as follows: CFP, n = 146; WT, n = 133; H150Y, n = 158; DMSO, n = 202; AK-1, n = 161; AGK2, n = 138; SIRT2H150Y, n = 230; SIRT2H150YΔNES, n = 200. Statistical Analysis. *C. elegans* data were analyzed by one-way ANOVA with correction for multiple testing by Tukey’s Multiple Comparison Test. Quantitative real-time PCR was performed as described (3) and analyzed by using Student’s t test. Statistical significance was assessed between data pairs with a one-tailed Student’s t test using Microsoft Excel following normality and equal variance tests with SigmaStat 3.5. All data were quantified based on hypotheses that were alternative to the null and significance was attributed by using an alpha level beginning at 0.05. P values, represented by asterisks, are indicated in corresponding figure legends.
Although AK-1 significantly reduced the number of inclusions (Fig. 2B), both large intranuclear (I and J) or small intra- and extranuclear inclusions (K and L) were observed and there was no change in the size distribution of Htt-positive inclusions (M). Reduction in number of inclusions (Fig. 2D and G, respectively) without an effect on inclusion size also occurred with AGK2 treatment (N) and expression of SIRT2H150Y (O). * * * ** * * *
**Fig. S2.** (A) SIRT2 protein expression after infection of primary striatal neurons with lentiviral vectors encoding SIRT2^WT or SIRT2^H150Y. (B) Dominant negative activity of deacetylase mutant SIRT2^H150Y. SIRT2 with an added nuclear exclusion signal (NES) expression was indistinguishable from SIRT2^WT, but exhibited higher proficiency for tubulin deacetylation. α-tubulin deacetylation conferred by heterologous expression of NES is significantly blocked by coexpression of SIRT2^H150Y in HEK293T cells (*P < 0.05).

**Fig. S3.** SIRT2 expression in neuronal cells and mouse brain tissues. SIRT2 tissue distribution in mouse brain. (A and B) SIRT2 is present in neuronal and non-neuronal cells in the adult mouse brain. Coronal sections (including the striatum) from 14-week-old wild-type and R6/2 brains from mice at 12 weeks of age were immunostained with antibodies to SIRT2 (red) and CNPase (an oligodendrocyte marker, shown as green in A) or NeuN (neuronal nuclei, shown as green in B); all nuclei were identified by counterstaining with TO-PRO-3 (blue). As ascertained in merged images in B, SIRT2 was found to be present in neuronal (white) and nonneuronal (pink) cells, and distributed throughout the neuropil and localized to white matter bundles (arrowhead). SIRT2 is heavily expressed in oligodendrocytes, as is shown by colocalization with CNPase in the white matter bundles of the striatum (A). (Scale bar: 50 μM.) (C) SIRT2 is present in striatal neurons in culture. (Upper) Double-labeling with NeuN confirms the expression of SIRT2 in neurons. SIRT2 immunoreactivity is present in CFP-, Htt^171-18Q-, and Htt^171-82Q-expressing cells. No apparent change in the levels or distribution of SIRT2 protein is observed in Htt^171-82Q cells (arrow indicates a polyglutamine inclusion). (Scale bar: 10 μM.) (D) Western blot analysis of SIRT2 levels in extracts prepared from neuronal cell-lines (NT2, PC12, ST14A, and SY5Y) and in cerebral cortex extracts from 2.5-month-old wild-type (WT) and transgenic HD (R6/2) mice. Two SIRT2-immunoreactive species of ≈43 kDa and ≈37 kDa are indicated by arrows. No change in SIRT2 expression is observed in HD animals (93 ± 21% of WT levels considering both ≈43 kDa and ≈37 kDa immunoreactive bands). rhSIRT2, recombinant human protein. (E) Approximately 43 kDa SIRT2-immunoreactive species are equivalently present in control (CFP), Htt^171-18Q-, and Htt^171-82Q-expressing rat primary striatal neurons (100 ± 10% of CFP control for 18Q and 109 ± 11% of CFP control for 82Q). (F) Fractionation of mouse brain extracts shows that only the slower-migrating ≈43 kDa SIRT2-immunoreactive species is present in the soluble fraction. p, insoluble fraction; s, soluble fraction.
Fig. S4. Mutant huntingtin-induced changes in gene expression are not corrected by SIRT2 inhibitor treatment. To assess the relationship between HD and AK-1 effects on gene expression, the fold changes for all significant probesets in the Htt171-82Q versus Htt171-18Q comparison were plotted against the comparison of AK-1-treated Htt171-82Q versus Htt171-18Q samples. Overall, the polyQ signature remains uncorrected by the drug treatment, as is shown by the nonobservance of points moving toward the x axis.

Fig. S5. AK-1 effects (10 μM) on metabolic gene expression as assessed by QPCR (A) and corresponding cholesterol levels in independent wild-type culture samples (B). AGK2 effects (10 μM) on metabolic gene expression as assessed by QPCR (C) and corresponding cholesterol levels in independent wild-type culture samples (D). (E and F) Heterologous expression of the dominant-negative SIRT2 deacetylase mutant SIRT2H150Y recapitulates decreases in the expression of sterol biosynthesis genes (E) and sterol levels (F) as compared with SIRT2WT expression. SIRT2WT expression had no effect on sterol biosynthesis genes (E) or sterol levels. *, P < 0.05. chol, cholesterol (see Materials and Methods).
Fig. 56. SIRT2 inhibitor effect on mutant huntingtin toxicity and sterol biosynthesis is extranuclear. Expression of SIRT2 containing an additional nuclear export signal (NES) blocks the neuroprotective effect of AK-1 on mutant huntingtin-expressing neurons (A), whereas nuclear localized SIRT2 (NLS) (B) does not. (C) Sterol levels in neurons expressing extranuclear SIRT2 are significantly higher than in those expressing nuclear localized SIRT2 (NLS). (*P < 0.05) chol, cholesterol (Materials and Methods).

Fig. 57. Extranuclear SIRT2 decreases the propensity of extranuclear endogenous SREBP-2. Expression of SIRT2 containing an additional nuclear export signal (NES) increases the percentage of endogenous nuclear SREBP-2 with respect to CFP-expressing neurons, whereas nuclear localized SIRT2 (NLS) increases extranuclear SREBP-2. (Scale bar: 5 μM.) *, P < 0.05.
**Fig. S8.** (A) Schematic representation of SREBP-2 regulatory domains of and experimental SREBP-2 mutants. The constitutively active truncation mutant (SREBP-2^{ACT}) comprises amino acids 1–458; the dominant-negative truncation mutant (SREBP-2^{NEG}) comprises amino acids 295–458 (primarily DNA binding domain). Both were expressed robustly in the nucleus of cultured striatal neurons (Inset). (Scale bar: 10 μM.) (B–D) As expected, SREBP-2^{ACT} increases sterol biosynthesis gene expression and cholesterol levels (B and D), whereas SREBP-2^{NEG} decreases them (C and D). (E) Expression of SREBP-2^{NEG} had no effect on the number of mutant Htt positive inclusions. (F and G) Overexpression of SREBP-2^{ACT} abrogates neuroprotection by SIRT2 inhibition conferred by AK-1 (F), AGK2 (G), and SIRT2^{H150Y} (H). A lentiviral vector encoding CFP is used as a coinfection control. *, P < 0.05. chol, cholesterol (Materials and Methods).

**Other Supporting Information Files**

[Dataset S1 (XLS)](dataset-links)
[Dataset S2 (XLS)](dataset-links)