

SIRT1 Activates MAO-A in the Brain to Mediate Anxiety and Exploratory Drive

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DOI 10.1016/j.cell.2011.10.054

SUMMARY

SIRT1 is a NAD⁺-dependent deacetylase that governs a number of genetic programs to cope with changes in the nutritional status of cells and organisms. Behavioral responses to food abundance are important for the survival of higher animals. Here we used mice with increased or decreased brain SIRT1 to show that this sirtuin regulates anxiety and exploratory drive by activating transcription of the gene encoding the monoamine oxidase A (MAO-A) to reduce serotonin levels in the brain. Indeed, treating animals with MAO-A inhibitors or selective serotonin reuptake inhibitors (SSRIs) normalized anxiety differences between wild-type and mutant animals. SIRT1 deacetylates the brain-specific helix-loop-helix transcription factor NHLH2 on lysine 49 to increase its activation of the MAO-A promoter. Both common and rare variations in the SIRT1 gene were shown to be associated with risk of anxiety in human population samples. Together these data indicate that SIRT1 mediates levels of anxiety, and this regulation may be adaptive in a changing environment of food availability.

INTRODUCTION

Anxiety is an emotional response to a perceived threat or challenge. This response is crucial for learning, memory formation, adaptation to the environment, and ultimately survival. Inability to regulate these emotional responses leads to various mental disorders. It is estimated that in a given year almost 26% of Americans age 18 or older suffer from a diagnosable mental disorder, many of which are various forms of anxiety (Kessler et al., 2005). Therapeutic treatment of depression

and anxiety disorders can be achieved by increasing the availability of serotonin and norepinephrine in the brain. A number of drugs have been developed for this purpose to inhibit monoamine oxidases (MAOIs) as well as to inhibit serotonin reuptake (SSRIs).

SIRT1 is an NAD-dependent protein deacetylase that was shown to play a role in numerous metabolic processes in many tissues, including brain (Chen et al., 2008). For example, SIRT1 was recently shown to act in the brain to reduce the production of A β amyloid peptide and ameliorate symptoms in a murine Alzheimer's disease model (Donmez et al., 2010). Two laboratories have also reported that SIRT1 affects learning and memory (Gao et al., 2010; Michán et al., 2010).

Here we report the investigation of the role of brain SIRT1 on mood and behavior. Our findings suggest a mechanistic relationship between anxiety and SIRT1 levels in the brain, which are driven by the deacetylation of a transcription factor regulating the monoamine oxidase A gene. Our results indicate that manipulation of brain SIRT1 activity may affect human mood in a predictable way, which may aid and encourage development of sirtuin drugs that cross the blood brain barrier.

RESULTS

SIRT1 Mutant Mice Have Altered Anxiety and Exploratory Drive

To evaluate the global impact of SIRT1 on brain physiology, we compared microarray gene expression profiles of brain tissues from brain-specific SIRT1 knockout mice (BSKO), which have the catalytic domain of this sirtuin (exon 4) deleted in the nervous system (Cohen et al., 2009) (Figure 1A), to those of wild-type (WT) littermates (NCBI accession number: GSE28790; Figures S1A–S1G and Table S1 available online). One of the most overrepresented categories of genes (as classified by the Gene Ontology database) (Ashburner et al., 2000) was one that determines behavior, specifically locomotor behavior and stress responses (Table S1).

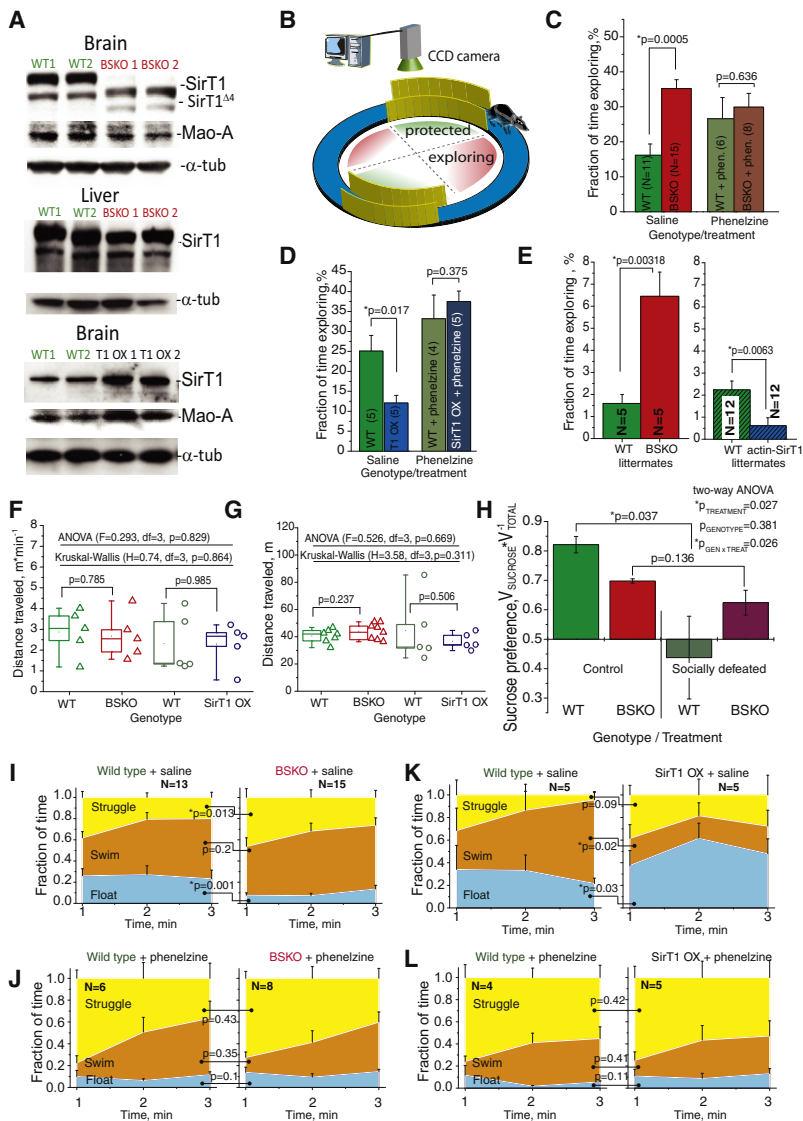


Figure 1. SIRT1 Affects Anxiety and Exploratory Drive

(A) BSKO mice that lack functional SIRT1 in the brain (these mice have exon 4 of SIRT1 deleted specifically in the brain) have lower levels of MAO-A, as shown by SDS-PAGE (top panel). Other tissues such as liver have normal SIRT1 (middle panel). Transgenic mice that overexpress SIRT1 in the brain (Sirt1 OX) have higher levels of MAO-A (lower panel). Typical SDS-PAGE blots of SIRT1 and MAO-A are presented here.

(B) Zero-maze test for anxiety and exploratory drive in rodents. Mice are naturally afraid of open spaces and prefer to be next to a wall or in an enclosed area. The fraction of time the animal spends in the open arms of the maze is indicative of the exploratory drive and anxiety of that animal.

(C) BSKO mice are less anxious and have enhanced exploratory drive, and this difference can be normalized by the MAOI phenezine. Also see Figures S1K and S1L. Mean \pm SEM is shown.

(D) SIRT1 OX mice have diminished exploratory drive, and this difference can be normalized by phenezine. Also see Figure S1M. Mean \pm SEM is shown.

(E) BSKO mice explore more and SIRT1 OX mice explore less than their littermates in open-field test paradigm. Also see Figure S1N. Mean \pm SEM is shown.

(F and G) BSKO and SIRT1 OX mice have similar activity during anxiety tests as their WT littermates. Digital videos of mice during corresponding tests (F—zero-maze, G—open-arena test) were analyzed, and distance traveled for each mouse was established. Box-plots and individual data points are presented here. Whiskers of box-plots represent 5%–95% data span. Box-plots present median, 25th, and 75th percentiles of data.

(H) BSKO mice are less susceptible to depression and anhedonia following a social defeat. Mean \pm SEM is shown.

(I–L) BSKO mice are resistant to depression, and SIRT1 OX mice are more susceptible. (I) BSKO mice are resistant to depression in Porsolt's test (spend less time floating); these differences can be normalized (J) by phenezine. (K) SIRT1 OX mice are more susceptible to depression, and this difference can be normalized (L) by phenezine.

Data presented as mean \pm SEM. p values are calculated using t test (in surface plots, area is used for p value calculation; Figures S1L–S1N contain complete statistical analysis of the data presented).

Based on these observations, we performed a number of specific tests to study the behavior of mice with altered SIRT1 levels in the brain. The first of these, the zero-maze, is a test designed to assess the exploratory drive of animals as well as their levels of anxiety (Shepherd et al., 1994). Mice are naturally anxious in open spaces and prefer to be next to a wall or in an enclosed area. Zero-maze consists of a circular, raised platform, two quadrants of which are protected and two quadrants of which are open (Figure 1B). Mice are filmed while in the maze, and their behavior is analyzed. BSKO mice explored the open arms of the radial maze almost twice as much as their WT littermates (Figures 1C and S1L). A similar anxiolytic effect and increase in exploration were achieved pharmacologically. Phenezine, a drug that inhibits MAO enzymes (Figure 1C), and fluoxetine, a serotonin reuptake inhibitor (Figure S1K), enhanced exploratory drive of WT mice to the level of BSKO mice but did little to the already overactive behavior of BSKO mice. This

experiment suggested that the difference in behavior of BSKO mice is unlikely to be due to some physical difference, such as muscle strength, because mood-modifying drugs could normalize exploratory behaviors. Additionally, these results provided an initial suggestion that SIRT1 affects behavior by altering serotonin levels in the brain. Interestingly, one of the genes that was identified by microarray analysis to have decreased expression in the brains of BSKO mice (Figure S1C) was monoamine oxidase A (MAO-A), which encodes an enzyme that regulates availability of serotonin in the brain.

Conversely, we found that SIRT1-overexpressing mice (SIRT1 OX mice) (Bordone et al., 2007), which have about twice as much brain SIRT1 protein as control mice (Figures 1A and S1J), had lower levels of exploratory drive compared to their WT littermates (Figures 1D and S1M). Similarly, treatment with phenezine equalized the exploratory drive of transgenic mice and their WT littermates (Figures 1D and S1M), again indicating that

differences in behavior evoked by SIRT1 dosage are driven by psychological changes.

Another assay for anxiety and exploration is the open-field test (Cunha and Masur, 1978), in which mice are put into a large circular arena, surrounded by walls. Anxiety in open spaces will force mice to spend most of their time next to the border of the arena. The fraction of time the mice spend exploring the center of the arena versus the edges can be used for quantification of rodent anxiety and exploratory drive. As with zero-maze tests, we found that BSKO mice spent more time in the center, demonstrating less anxiety-like behaviors and more exploratory drive than their WT littermates (Figure 1E). Conversely, SIRT1 OX mice displayed more anxious behavior compared to control littermates (Figures 1E and S1N).

We previously reported that BSKO mice have elevated physical activity (Cohen et al., 2009), when measured by a free running wheel experiment. Therefore, we assessed the activity of BSKO mice, SIRT1 OX mice, and their WT littermates by measuring their average distance traveled in both zero-maze (Figure 1F) and open-field tests (Figure 1G) and found no statistically significant differences. Thus, when mice with altered SIRT1 are introduced into a novel environment, their physical activity is not changed compared to WT, therefore corroborating that altered behavior of these transgenic mice is independent of their physical activity.

Anxiety often manifests accompanied by depression. We therefore used the “social defeat” paradigm and the Porsolt’s test (forced swim test) to measure susceptibility of animals to depression. In the social defeat paradigm, the state of depression is induced in experimental male subjects after repeated exposure to stronger, more aggressive dominant males. Socially defeated males exhibit a number of changes in their behavior and physiology (Von Frijtag et al., 2000), including anhedonia, a manifestation of depression-like symptoms measured by decrease in preference of a sucrose solution over normal drinking water. We subjected WT and BSKO mice to social defeat and found that SIRT1 BSKO mice were more resistant to depression than their WT littermates (Figure 1H). This study must be interpreted with caution, as BSKO mice had a somewhat lower baseline preference for sucrose, which could be explained by alterations in their metabolism (Cohen et al., 2009), taste, or olfaction.

In the forced swim test, naive mice are placed into a tall cylinder filled half-way with water, where they will swim around and try to find an escape by attempting high jumps, a behavior called “struggle.” Over time, however, mice cease their attempts to escape and float passively, indicative of a depression-like state. BSKO mice were less susceptible to depression (Figure 1I), as they attempted escape more often and spent less time floating. Pharmacological inhibition of MAO enzymes by phenelzine normalized behavior of BSKOs and their WT littermates (Figure 1J). Conversely, SIRT1 OX mice were more susceptible to depression (Figure 1K), and again phenelzine treatment normalized their behavior (Figure 1L).

We hypothesized that the SIRT1 effect on behavior might be mediated by changes in the levels of serotonin and perhaps other neurotransmitters that affect anxiety (Nordquist and Orelund, 2010). Thus we performed high-pressure liquid chromatography (HPLC) analysis of whole brains and quantified

concentrations of noradrenalin, dopamine, and serotonin of WT, BSKO, and SIRT1 OX mice. BSKO mice had significantly higher levels of serotonin (Figure 2A) and noradrenalin (Figure 2B) than their WT littermates, but no significant difference in the levels of dopamine (Figure 2C) was detected. Additionally, BSKO animals had significantly lower levels of 5-hydroxyindoleacetic acid (5-HIAA, Figure 2D). SIRT1 OX mice had reciprocal levels of serotonin and 5-HIAA compared to BSKO—lower levels of serotonin and higher levels of 5-HIAA (Figures 2E–2H). Similar findings were made in subsections of the brains obtained by dissection (Figures S2A–S2J).

5-HIAA is an oxidation product of serotonin (Akillu et al., 2009), and MAO-A is the major enzyme in the brain that converts serotonin into 5-HIAA (Figure 2I). Treatment of WT animals with the MAO-A inhibitor phenelzine resulted in a similar pattern of changes of serotonin (Figure 2J) and 5-HIAA (Figure 2K) as in BSKO brains. Our findings therefore suggest that there is diminished MAO-A activity in BSKO mice and elevated MAO-A activity in SIRT1 OX mice. We verified this hypothesis by directly measuring specific MAO-A activity in the mitochondrial extract (Hampp et al., 2008) from the whole brains of BSKO animals and their WT littermates and found a 12.45% decrease ($p = 0.0059$) in specific MAO-A activity in brains of SIRT1 knockout animals (Figure S2K).

MAO-A Transcription Is Controlled by SIRT1 In Vivo and In Vitro

To further address how SIRT1 controls MAO-A, we measured by SDS-PAGE the abundance of this enzyme in the brains of WT and mutant mice. BSKO mice had lower levels of MAO-A in their brains (Figures 1A, S1H, and S1I), whereas SIRT1 OX mice had higher levels (Figures 1A and S1J). It is known that the amygdala is a part of the brain particularly involved in anxiety and mood modulation, therefore we also measured abundance of MAO-A in this region and again found that SIRT1 OX mice had higher levels of MAO-A protein and BSKO mice had lower levels relative to WT littermates (Figure S2L).

MAO enzymes are mitochondria-bound proteins, whereas SIRT1 is generally nuclear (Prozorovski et al., 2008), suggesting that SIRT1 regulates MAO-A abundance at the level of transcription. Quantitative real-time PCR analysis showed that MAO-A mRNA abundance is lower in brains of BSKO mice (Figure 2L) and higher in brains of SIRT1 OX mice (Figure 2M) compared to their WT littermates. We obtained similar results for the amygdala (Figure S2M). These experiments suggested that SIRT1 is an activator of MAO-A transcription in vivo. To further confirm this hypothesis, we performed chromatin immunoprecipitation (ChIP) experiments and found that SIRT1 binds to the MAO-A promoter in close proximity to the ATG start codon but not to a region 2 kilobases (kb) upstream (Figure 3A). To demonstrate the specificity of the ChIP experiment, we performed identical experiments in SIRT1 null mice and found no enrichment by SIRT1 antibody of any DNA regions (Figure 3A).

To begin to study the mechanism by which SIRT1 activates transcription of MAO-A, we transfected mouse neuroblastoma cells (N2A) with a plasmid that expresses human SIRT1 via the CMV promoter. Note that human SIRT1 is slightly larger than endogenous mouse SIRT1, giving rise to a slower-migrating band on the western blot (Figure 3B). A MAO-A

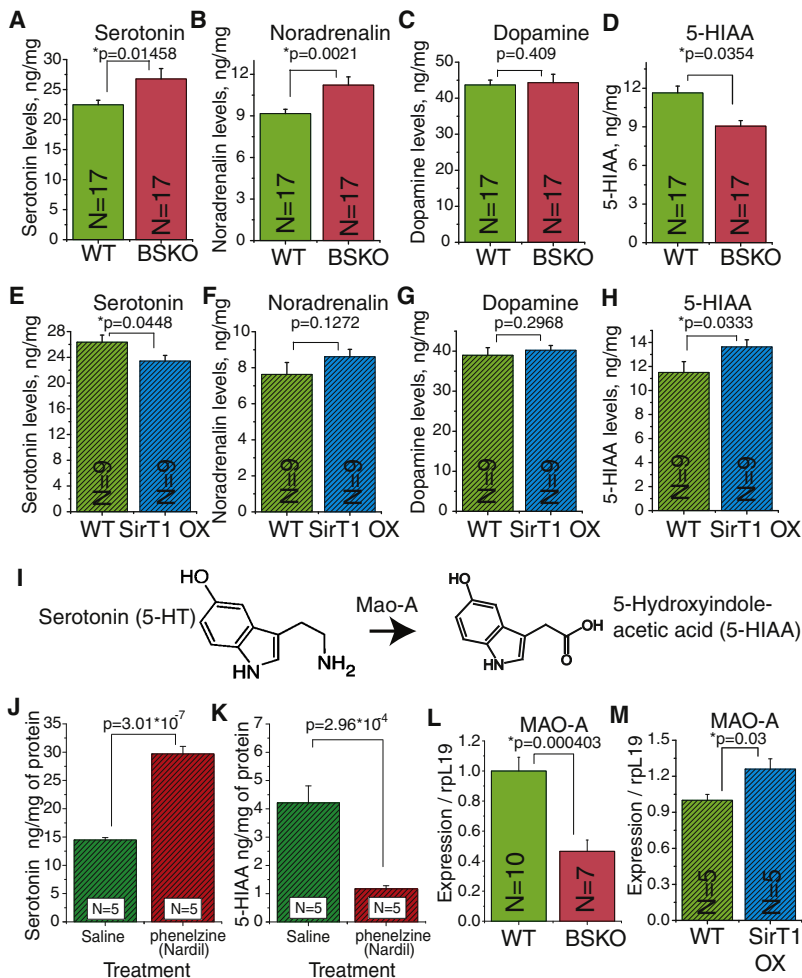


Figure 2. SIRT1 Regulates Levels of Neurotransmitters

(A and B) BSKO mice have higher levels of (A) serotonin and (B) noradrenalin in the brain than their littermates. (C) Dopamine is not statistically different in BSKO mice. (D) BSKO mice have lower levels of 5-HIAA. (E–G) SIRT1 OX mice have lower serotonin (E) and similar noradrenalin (F) and dopamine (G). (H) SIRT1 OX mice have higher levels of 5-HIAA. (I) MAO-A is the major enzyme in the brain that converts serotonin into 5-HIAA. (J and K) Phenelzine (MAOI) treatment increases serotonin (J) and decreases serotonin degradation product 5-HIAA (K) levels in the brains of WT mice, similar to the effect of SIRT1 deletion. (L and M) RT-PCR indicates lower levels of MAO-A mRNA in the brains of BSKO mice (L) and higher levels in SIRT1 OX mice (M). (A–H and J–M) Data presented as mean \pm SEM. p values are calculated using t test. See also Figure S2.

promoter-luciferase construct (Hampp et al., 2008) was transfected simultaneously with the SIRT1-overexpressing plasmid or a control lacZ-expressing plasmid, revealing that enforced expression of SIRT1 activated transcription of luciferase (Figure 3B). In order to reduce SIRT1 function, we expressed short hairpin RNAs targeted against SIRT1 and observed a decline in MAO-A promoter activity as compared to a scrambled control shRNA (Figure 3C). These data further indicated that SIRT1 directly regulates activity of the MAO-A promoter.

To investigate whether enzymatic activity of SIRT1 is important for its ability to activate the MAO-A promoter, we expressed a mutant SIRT1 allele, where histidine at position 363 is substituted for tyrosine (H363Y), which reduces the enzymatic activity (Pfeister et al., 2008). We observed that this mutation also reduced the ability of SIRT1 to activate the MAO-A promoter (Figure 3D and Table S2), suggesting that SIRT1 enzymatic activity is required for complete activation of the MAO-A promoter.

NHLH2 Is Deacetylated by SIRT1 to Activate Expression of MAO-A

The MAO-A-luciferase reporter contained a promoter sequence extending 1.1 kb upstream of the MAO-A translation start site

(Hampp et al., 2008), so we hypothesized that one of the transcription factors that binds to this sequence is likely the target of SIRT1. To define the SIRT1-responsive promoter region, we first cloned four overlapping 300 bp subsections of the original 1.1 kb promoter into the luciferase vector and transfected each construct into N2A cells with normal, knocked down, or overexpressed SIRT1 (Figure 4A, left panel). Consistent with ChIP, the most ATG-proximal 300 bp fragment displayed full promoter activity and SIRT1 responsiveness. Next we subcloned four overlapping 90 bp subregions of this 300 bp fragment into the luciferase reporter and tested activity and SIRT1 responsiveness

in N2A cells (Figure 4A, right panel, Figure 4B). The two middle and overlapping 90 bp fragments had the strongest signal, as well as responsiveness to SIRT1. Using bioinformatics tools (Marinescu et al., 2005), we searched for transcription factors that could bind these pieces of DNA and identified two sites for NHLH1 and NHLH2 (Brown et al., 1992) (Figure 4B), which are related brain-specific helix-loop-helix transcription factors (Brown et al., 1992) (Figure S3A). Both NHLH2 (Coyle et al., 2002; Good et al., 2008) knockout mice and BSKO (Cohen et al., 2009) mice show changes in physical activity consistent with the possibility that NHLH2 is a SIRT1 target.

We thus generated a series of artificial luciferase reporter constructs containing repeats of CGCAGCTGCG (NHLH2 consensus-binding sequence, Figure S3A). Although this construct was SIRT1 responsive, its activity was rather low. Interestingly, the 90 base SIRT1-responsive fragments also contained binding sites of the ubiquitous transcription factor SP1, and incorporation of synthetic SP1-binding sites between NHLH2 sites created a potent promoter, which responded more strongly to SIRT1 (Figure 4C). SP1 sites alone were not sufficient to provide responsiveness to SIRT1 (Figure 4C and Tables S3A–S3D).

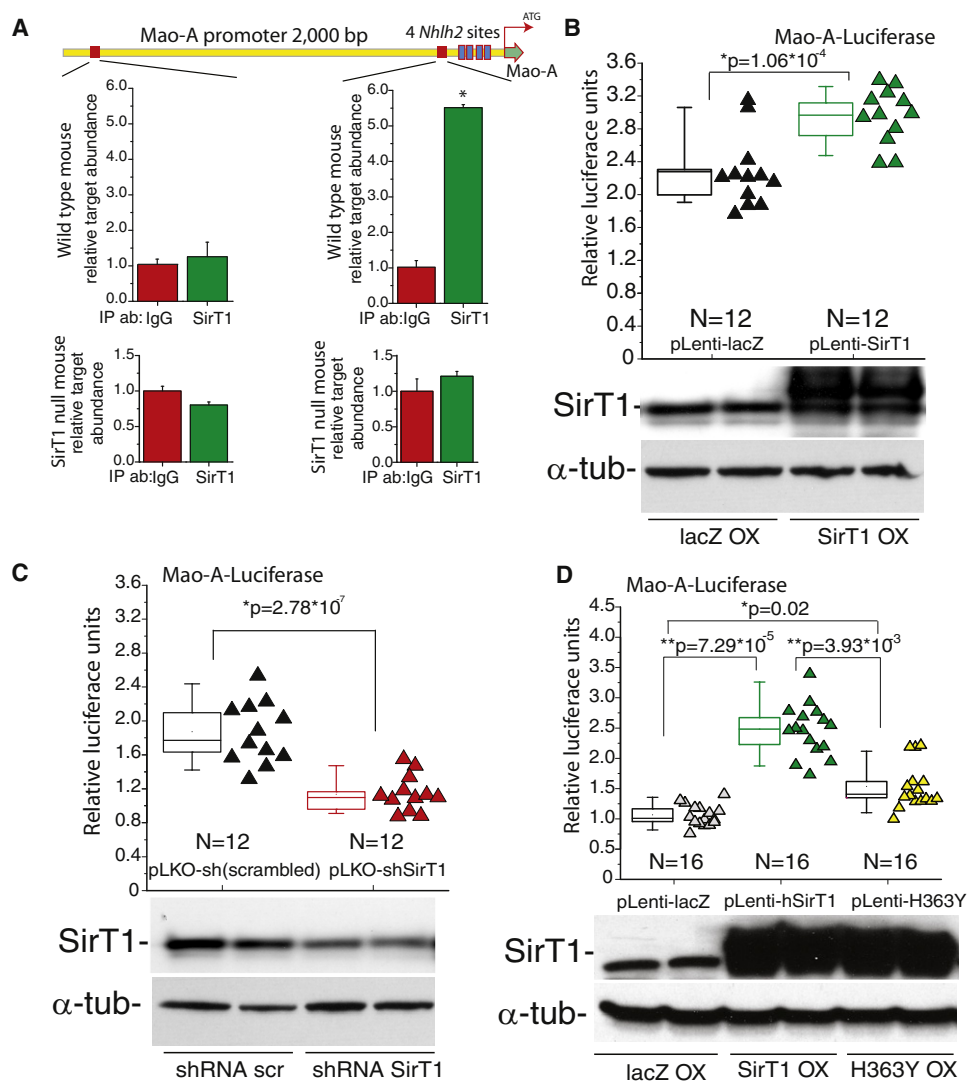


Figure 3. SIRT1 Directly Activates MAO-A Transcription

(A) Chromatin immunoprecipitation reveals the presence of SIRT1 on the MAO-A promoter-proximal DNA but not 2 kb upstream or anywhere in SIRT1 null mice. Data presented as mean \pm SEM. p values are calculated using t test.

(B) SIRT1 overexpression activates MAO-A transcription as measured by a luciferase reporter.

(C) Knockdown of endogenous SIRT1 by short hairpin RNA reduces MAO-A transcription.

(D) SIRT1 enzymatic activity is required for its ability to activate the MAO-A promoter. The H363Y mutation largely suppresses ability to activate MAO-A transcription (Table S2 presents complete statistical analysis of data shown using ANOVA).

(B–D) Box-plots present median, 25th, and 75th percentiles of data. Whiskers present 5th and 95th percentiles of data, with actual data points on the right. p values are calculated using t test.

To confirm that NHLH2 is indeed a bona fide transcriptional factor that responds to SIRT1 on the MAO-A promoter, we mutated both NHLH2 sites in the 1.1 kb MAO-A promoter by substituting eight bases in each NHLH2-binding site with a string of adenosines, so that spatial relationships in the promoter were not disturbed, and found that the resulting mutant promoter lost activity and responsiveness to SIRT1 (Figure 4D).

The hypothesis that SIRT1 acts on NHLH2 to modulate MAO-A activity predicts that SIRT1 and NHLH2 would interact physically or at least be part of the same molecular complex. To verify this, we performed a series of coimmunoprecipitation

(co-IP) experiments (Figures S3B–S3E). We first immunoprecipitated NHLH2 from the brain tissue of WT mice and found that SIRT1 coprecipitates with it (Figure S3B). Reciprocally, when SIRT1 was immunoprecipitated from the mouse brain lysate, NHLH2 coprecipitated with it (Figure S3C).

We next coexpressed human *myc*-tagged NHLH2 and human V5-tagged SIRT1 in N2A cells and found that these proteins also coimmunoprecipitate (Figures S3D and S3E). Additionally, we found that expressing human *myc*-tagged NHLH2 activated the 1.1 kb MAO-A promoter by about 5-fold (Figure S4A, see also Figure 5D, second lane). At these high levels of NHLH2,

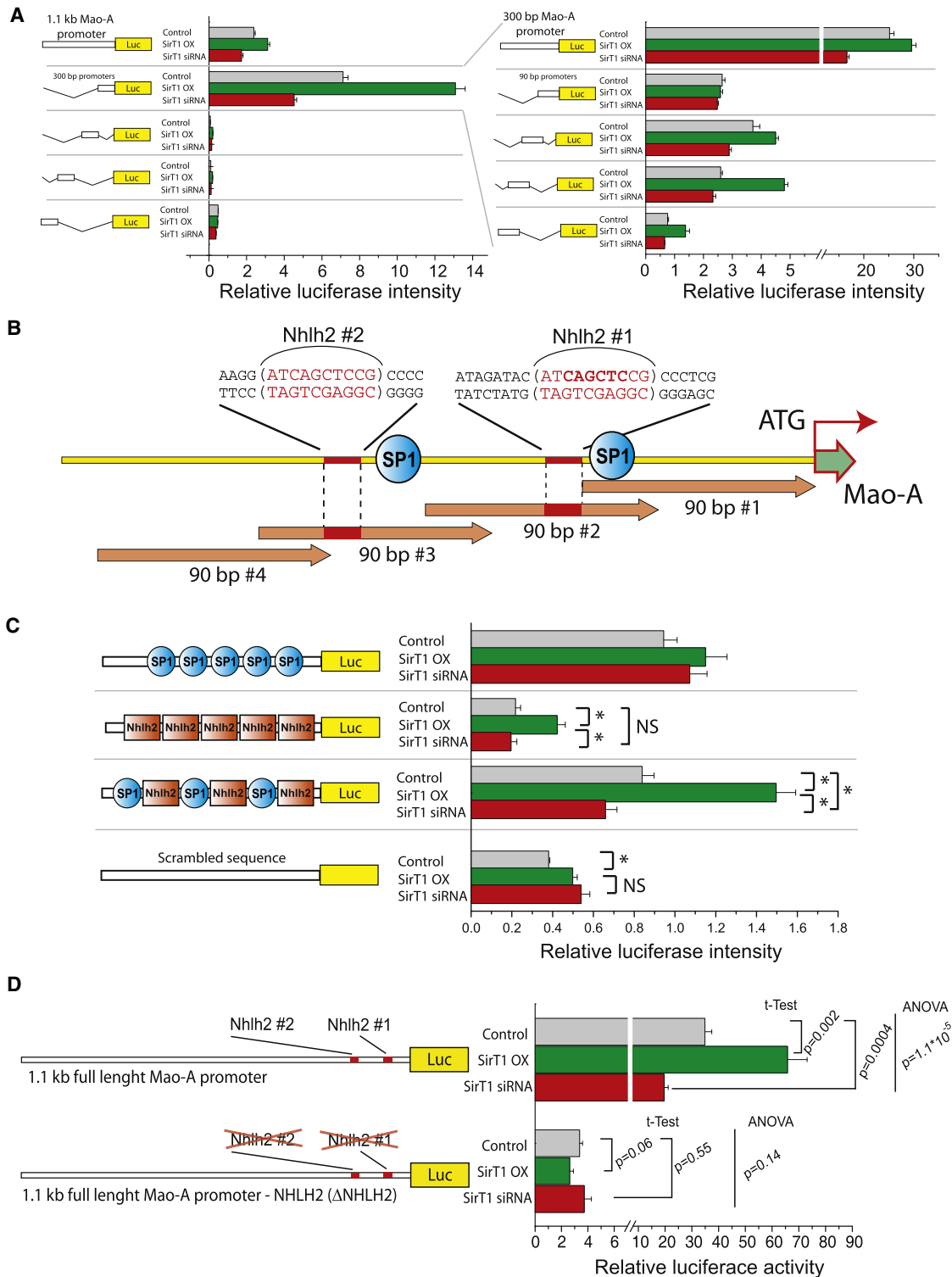


Figure 4. Identification of NHLH2 Sites as SIRT1 Response Elements in the MAO-A Promoter

(A) Fragmentation of the MAO-A promoter identified candidate transcriptional factors that may be regulated by SIRT1. The 1.1 kb MAO-A promoter was segmented into four 300 bp fragments and cloned in front of luciferase. Only the most ATG-proximal fragment had promoter activity as well as SIRT1 responsiveness (left panel). This 300 bp fragment was segmented into four 90 bp overlapping fragments, with the two middle fragments possessing promoter activity and SIRT1 responsiveness (right panel). Mean \pm SEM is shown.

(B) Bioinformatics identifies NHLH1/2 sites in the 90 bp SIRT1-responsive fragments. See also Figure S3A.

overexpression of SIRT1 did not further activate the *MAO-A* promoter (Figure S4B), suggesting that NHLH2 and SIRT1 activate *MAO-A* via the same pathway.

We proceeded to study the mechanism by which SIRT1 regulates NHLH2 activity on the *MAO-A* promoter. First, we investigated the acetylation status of NHLH2 in vivo. We found that NHLH2 is hyperacetylated in the brains of BSKO mice and hypoacetylated in the brains of SIRT1 OX mice as compared to corresponding WT littermates (Figure 5A), providing further evidence that SIRT1 deacetylates NHLH2 and thus increases its transcriptional activity. We next purified human NHLH2-myc from N2A cells, as well as cells that either overexpressed SIRT1 or had SIRT1 suppressed by shRNA or nicotinamide, and subjected it to liquid chromatography-tandem mass spectrometry (LC-MS/MS). We found that a single residue, K49, was acetylated (Figures 5B and 5C), but only when the activity of SIRT1 was suppressed by shRNA or nicotinamide—no acetylation of NHLH2 was detected in samples from cells that overexpressed SIRT1 or in control cells. It should be noted that this experiment was repeated three times, and similar numbers of counts for the tryptic peptide containing K49 were detected for each sample. These data show that NHLH2 can be acetylated on K49, and that SIRT1 is capable of deacetylating this residue.

We next expressed human NHLH2 with K49 mutated to arginine (K49R) or glutamine (K49Q) and investigated the activity of these mutant transcription factors on the 1.1 kb *MAO-A* promoter (Figure 5D). WT NHLH2 upregulated *MAO-A* transcription almost 5-fold, but K49R and K49Q mutants only 4- and 3-fold, respectively (Figure 5D), suggesting that K49 is an important residue for determining NHLH2 activity on the *MAO-A* promoter. Critically, we found that reduction of SIRT1 abundance by shRNA decreased activity of WT NHLH2 by 57%. However, the same reduction of SIRT1 resulted in only a 14% activity reduction of K49Q mutant and a 28% reduction in the K49R mutant (Figure 5D). These results suggest that deacetylation of K49 by SIRT1 regulates NHLH2 activity. The residual response that is observed in K49 mutants may indicate that a minor component of NHLH2 activation occurs through binding of SIRT1 or deacetylation at secondary sites.

To further prove that SIRT1 directly deacetylates NHLH2, we performed in vitro acetylation/deacetylation assays. After codon optimization, we expressed and purified recombinant GST-SIRT1 from bacterial host designed for protein expression. We also expressed and purified NHLH2-myc (WT and K49R mutant) from N2A cell culture. First we in vitro acetylated NHLH2 with the HAT domain of p300 (Figure 5E, lanes 4 and 5, as compared to unacetylated NHLH2, lanes 2 and 3). WT protein was more acetylated as compared to the K49 mutant (compare lanes 4 and 5), indicating that K49 is a major site of acetylation in vitro (but not the only site). After washing and repurification of acetylated NHLH2, we subjected these molecules to recombinant SIRT1 (Figure 5E, lanes 6 and 7). WT NHLH2 was deacetylated to

a much higher degree than the K49R mutant (on Figure 5E, compare the large decrease in acetylation between lanes 4 and 6 for WT with the smaller change in acetylation between lanes 5 and 7 for K49R). These data further supported the claim that SIRT1 directly deacetylates NHLH2 on K49, which activates its transcriptional activity on the *MAO-A* promoter to cause changes in behavior.

SNPs in the *SIRT1* Gene Are Associated with Risk of Anxiety in Humans

To determine whether *SIRT1* is associated with psychiatric disorders in humans, we genotyped 14 single-nucleotide polymorphisms (SNPs) encompassing the *SIRT1* gene in a random sample of 3,420 men and women between the ages of 35 and 66 from the City of Lausanne, Switzerland (PsyCoLaus study; Preisig et al., 2009). All participants were assessed using a semi-structured interview (Nurnberger et al., 1994), and diagnoses were assigned according to DSM-IV (American Psychiatric Association, 1994). Participants also completed the Trait Subscale of the State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970), which provides a measurement of the overall level of persistent anxiety. We tested SNPs in *SIRT1* for association with specific anxiety disorders, the overall level of anxiety, and depression (Figures 6A–6D). We observed association between SNPs in *SIRT1* and any anxiety disorder (rs10997870), panic disorder (rs12778366 and rs10997870), and social phobia (rs12778366), which survived both Bonferroni and permutation-based ($n = 10,000$ permutations) multiple testing corrections ($p_{\text{corrected}} < 0.05$). A trend of association was also observed between rs12413112 and major depressive disorder; however, this result did not survive Bonferroni correction (Figure 6D).

We attempted to replicate our strongest human association finding for this gene, SNP rs10997870 with the specific anxiety phenotype of panic disorder, in a comparable independent sample. The Virginia Adult Twin Study of Psychiatric and Substance Use Disorders (VATSPSUD) is a longitudinal community study of over 9,000 adult Caucasian twins born in Virginia (Kendler et al., 2001). Lifetime psychiatric diagnoses were obtained via face-to-face or telephone-administered psychiatric interviews based on the Structured Clinical Interview for DSM-III-R (SCID) (Spitzer et al., 1992). We genotyped rs10997870 using the TaqMan method (Livak, 1999) in 1,398 independent subjects from a subset of the VATSPSUD previously selected for molecular genetic studies based upon genetic risk for internalizing disorders (Hettema et al., 2008). Among these, we identified 117 subjects with lifetime panic disorder (the cases) and 675 controls with no lifetime mood or anxiety disorders for analysis. Case-control analysis revealed a predisposing association between rs10997870 (the G allele as in the Lausanne study) and risk of panic disorder (freq.[cases] = 0.397, freq.[controls] = 0.329, $\chi^2 = 4.182$, $p = 0.041$), confirming our initial discovery (Figure 6D).

(C) Artificial promoters of five repeats of the NHLH2-binding site show SIRT1 responsiveness. NHLH2 sites interspersed with SP1-binding sites create a more potent promoter also responsive to SIRT1. The promoter with SP1 sites only is not responsive to SIRT1 (top construct). See also Table S3 for complete statistical analysis of data presented here.

(D) The full-length *MAO-A* promoter with mutated NHLH2-binding sites loses activity and responsiveness to SIRT1.

(C and D) Data presented as mean \pm SEM. p values are calculated using t test and ANOVA (* denotes $p < 0.05$). See also Figure S3.

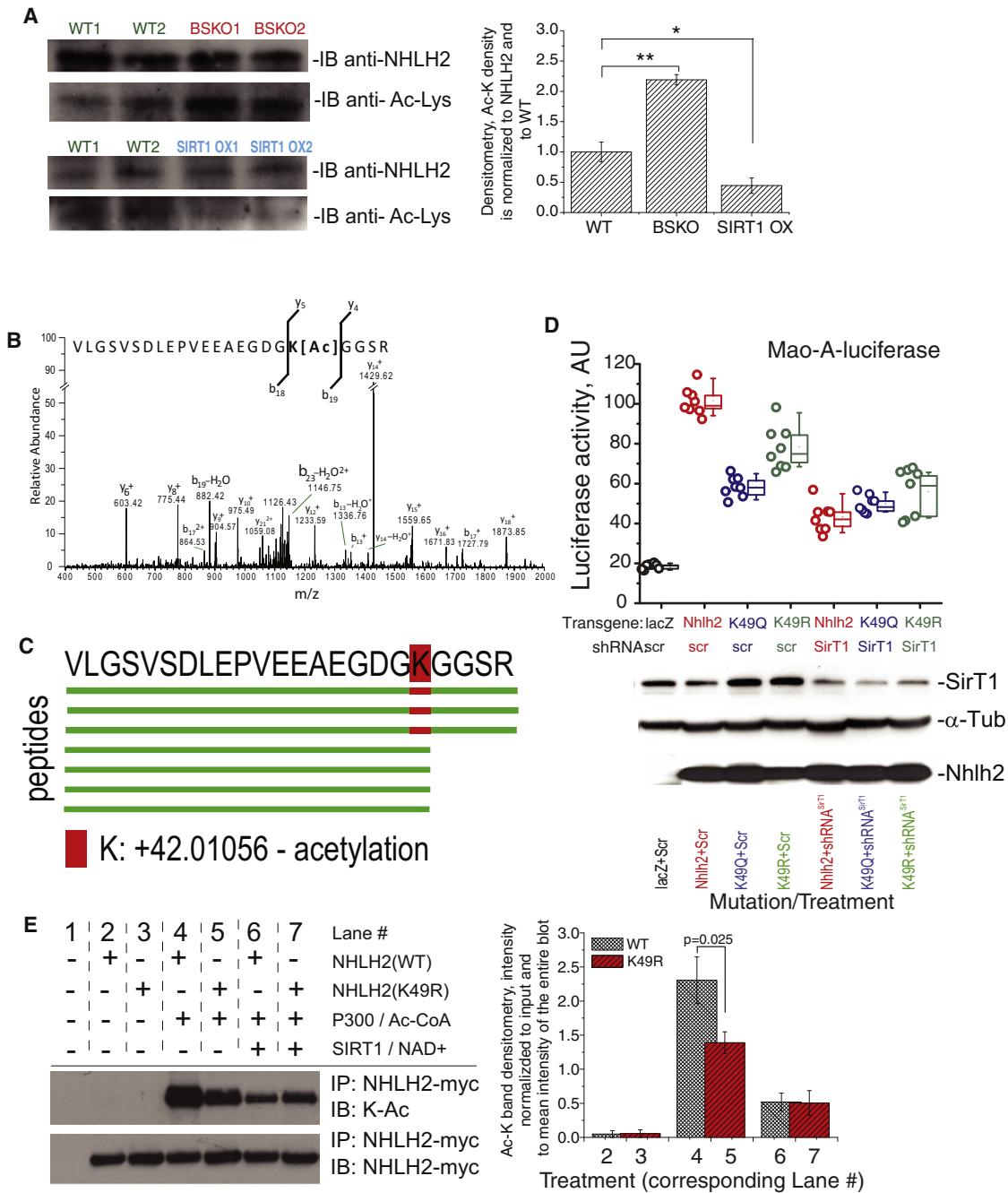


Figure 5. NHLH2 Can Activate MAO-A, and Lysine 49 of NHLH2 Is Deacetylated by SIRT1

(A) Left panel, NHLH2 is hyperacetylated in brains of BSKO mice and hypoacetylated in brains of SIRT1 OX mice. Typical SDS-PAGE is shown. Immunoprecipitated NHLH2 from brain lysates of WT and transgenic mice was probed with anti-NHLH2 antibody (loading control) or anti-Ac-K antibody. Each lane represents one mouse, and three mice of each genotype were tested. Right panel, quantification of the data presented in the left panel. Blots were quantified using the densitometry function in Image-J, NIH. At least three animals were used per genotype. * denotes $p < 0.05$, ** denotes $p < 0.01$. Intensity of Ac-K bands was normalized to intensity of NHLH2 bands, and all the data were normalized to WT signal strength. Mean \pm SEM is shown.

(B) Tandem mass spectrometry (LC-MS/MS) analysis of NHLH2 identified lysine 49 to be acetylated (K49). Typical ion fragmentation diagram is presented. Fragment ions were consistent with acetylation at site K49 with ion series shifting by 42 Da starting at b_{19} from the N terminus of the peptide and y_5 from the C terminus, which corresponds to acetylation of K49.

(C) Acetylated peptides on K49 are also resistant to Trypsin digestion. K49 was the only acetylated residue we identified. The experiment was repeated three times, and an identical number of acetylated peptide counts was identified for each sample.

(D) NHLH2 overexpression (left two lanes) activates 1.1 kb MAO-A promoter in N2A; also see Figure S4A. K49 residue was mutated to arginine (K49R) or glutamine (K49Q), and these mutant proteins or lacZ were expressed in N2A cells also transfected with a control shRNA (Scr) or a SIRT1 shRNA (shRNA^{Sirt1}).

A recent independent study conducted in Japanese subjects provides confirmatory evidence for our findings (Kishi et al., 2010). In this Japanese study, an association between another *SIRT1* SNP, rs10997875, and major depressive disorder was observed. This is relevant because the *SIRT1* SNP showing association in our study (rs10997870) is in strong linkage disequilibrium (LD) with rs10997875 in both Japanese ($R^2 = 96$) and European ($R^2 = 93$, Figure 6E) populations (Thorisson et al., 2005), thereby providing additional evidence for an association between a *SIRT1* haplotype encompassing these SNPs and psychiatric disorders. Further proof of a role for *SIRT1* in mood regulation in humans was provided recently by Abe et al. (2011), who showed that expression levels of *SIRT1* in white blood cells are associated with certain mood disorders.

To further explore the relationship between *SIRT1* genetic variation and mood disorders, we sequenced *SIRT1* exons in the 1,952 individuals from the PsyCoLaus study and investigated rare nonsynonymous (amino acid-changing) variants for association with anxiety. We found that carriers of rare nonsynonymous variants in *SIRT1* (Figure S5A) were more likely to have “any anxiety disorder” (panic disorder, agoraphobia, social phobia, or generalized anxiety disorder; with 43.1% of carriers having an anxious phenotype compared to 31.6% of the group as a whole; $p = 0.015$; Figure S5B). It is noteworthy that the majority of nonsynonymous mutations were located in the C’ or N’ terminus of the protein (Figure S5A) far from the catalytic domain, suggesting that the mutations may decrease interaction with another protein such as the inhibitor DBC1 (Kim et al., 2008; Zhao et al., 2008) or destabilize an internal negative regulatory domain. To investigate the mechanistic link between these mutations and *SIRT1* activity, we introduced two of the most frequent mutations in the N terminus (S14P and P37L) into our overexpression plasmid and tested the ability of these two mutant proteins to activate the MAO-A-luciferase reporter. We found that both S14P and P37L *SIRT1* mutant proteins (Figure 6F) had enhanced ability to activate MAO-A compared to WT (Figure 6G). We also tested activity of S14P and P37L mutants *in vitro*. Indeed, recombinant *SIRT1* enzyme (Figure S5C) with S14P and P37L mutations had increased activity compared to WT (Figure S5D). These data reinforce the hypothesis that *SIRT1* controls anxiety and mood disorders in humans, and that the mechanism of this control might be similar to the one we described in rodents.

DISCUSSION

We show that brain *SIRT1* influences anxiety and behavior and describe a mechanism for this effect (Figure S6A). *SIRT1* drives

anxiety-like behavior and inhibits exploratory behavior in mice by deacetylating the brain-specific transcription factor NHLH2 on lysine 49, which increases its activity on the MAO-A promoter. Because MAO-A degrades serotonin, increases in MAO-A reduce levels of this neurotransmitter, leading to anxiety and depression (Nordquist and Oreland, 2010). Indeed MAO-A inhibitors normalize anxiety differences in mice with altered levels of brain *SIRT1*. Genetic analyses of human *SIRT1* polymorphisms in independent cohorts of people suggest that the role of this sirtuin in modulating anxiety and mood is conserved.

It is intriguing that *SIRT1* may link nutritional status of animals to their behavior. Over the course of evolution, animals have faced variable periods of food availability, and the ability to respond to these fluctuations would be selected for. Below we discuss two scenarios, one short term (typical calorie restriction, CR) and the other long term (acting on intergenerational time-scales), to demonstrate the benefit of having mood and exploratory drive regulated by food availability.

CR is known to increase physical activity in laboratory rodents in their home cages (Weed et al., 1997), and *SIRT1* in the brain appears to be at least partially responsible for this increase (Chen et al., 2005). Although CR was reported not to change anxiety of rodents (Minor et al., 2008), we reinvestigated this using the more sensitive zero-maze paradigm and found CR mice (Figures 7A and 7B) to be more anxious than their ad libitum fed littermates (Figures 7C and 7D). The increase in activity observed in home cages has been interpreted to represent an increase in the urge to forage. However, we observed that CR mice traveled distances similar to those of ad libitum counterparts inside the zero-maze apparatus (Figure 7E). We suggest that the major behavioral impact of CR is to increase anxiety, perhaps to protect animals during foraging. The previously observed increase in activity in home cages may actually reflect this heightened anxiety-like state.

To address long-term food scarcity, we now consider the classical three-species food chain, described by the Lotka-Volterra model (Figures 7F and S6B). The number of species (food, species of interest [e.g., mice], and predators of that species) will oscillate in the order depicted on Figure 7F (also see Figure S6). The rise of food will be followed by the rise of the species of interest, followed by the rise of predators of that species. The ability to navigate these cycles and dynamically adapt will be strongly selected for in the species of interest. This cycle (Figure 7F) can be roughly split in two halves: On the left, food is abundant, the population of the species of interest increases, and predation is low (Figure 7F, green shaded area). On the right,

SDS-PAGE blots show *SIRT1* knockdown in cells (right three treatments). Activation of 1.1 kb MAO-A promoter in N2A cells shows that knockdown of *SIRT1* reduces activity of WT NHLH2 by 57% ($*p = 1.46 \cdot 10^{-11}$), whereas activities of K49Q and K49R are reduced by 14% ($*p = 8.33 \cdot 10^{-4}$) and 28% ($*p = 4.04 \cdot 10^{-4}$), respectively. Box-plots present median, 25th, and 75th percentiles of data. Whiskers present 5th and 95th percentiles of data, with actual data points on the left. p values are calculated using t test.

(E) Left panel, anti-acetyl lysine blot showing *SIRT1* deacetylation of K49 of NHLH2 *in vitro*. Purified recombinant NHLH2-myc (from N2A cells) was bound to anti-myc beads, washed, and acetylated with the HAT domain of p300 (lanes 4 and 5, as compared to lanes 2 and 3). After washing and repurification of acetylated NHLH2, purified GST-*SIRT1* (from *E. coli*) was added (lanes 6 and 7). WT NHLH2 was deacetylated to a much higher degree than K49R mutant (compare the large decrease in acetylation between lanes 4 and 6 for WT to the smaller change in acetylation between lanes 5 and 7 for K49R). Right panel, quantification of the data presented on the left panel. Blots were quantified using the densitometry function in Image-J, NIH. Intensity of Ac-K bands was normalized to intensity of NHLH2 bands. Mean \pm SEM is shown.

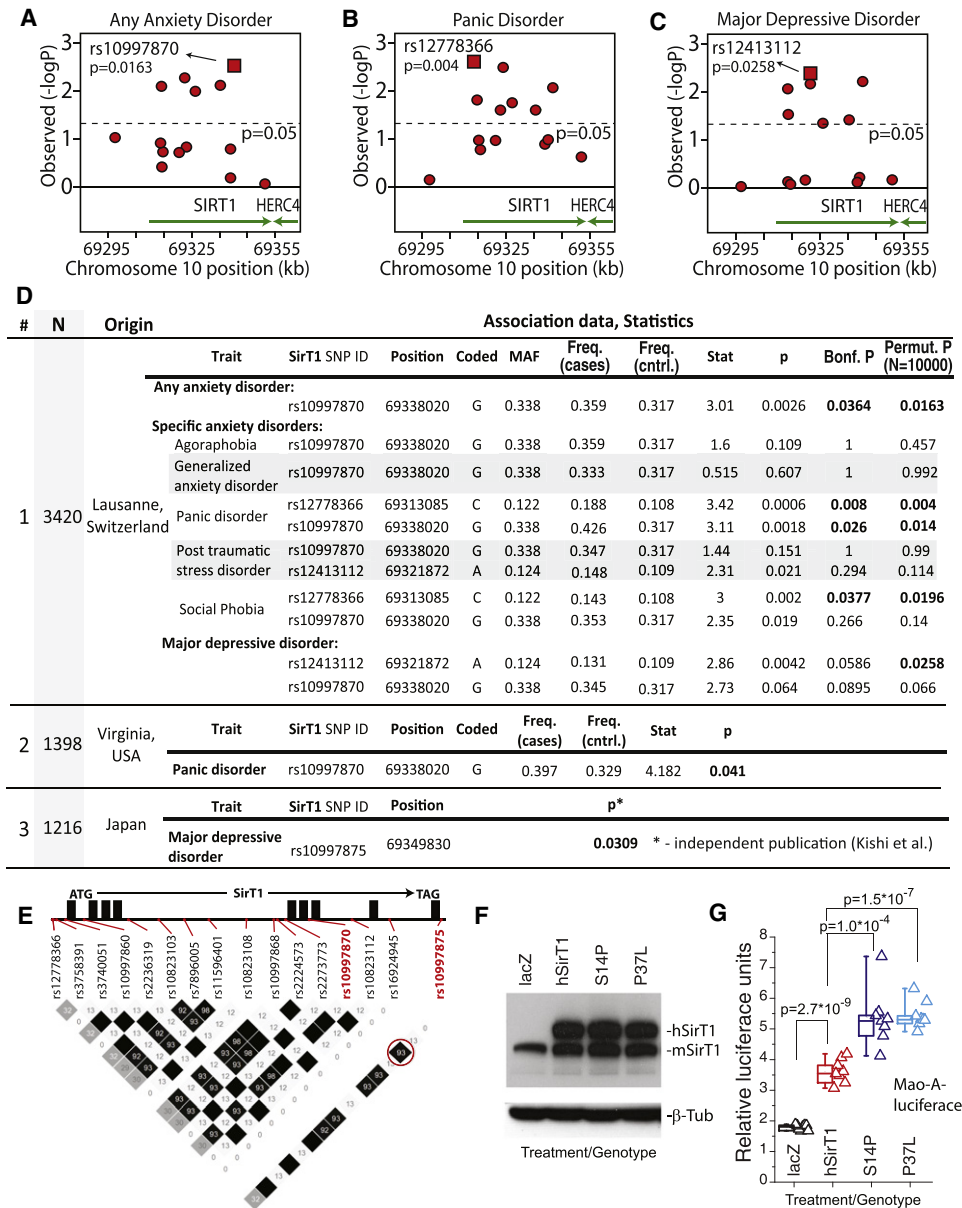


Figure 6. Genetic Variants in the *SIRT1* Gene Are Associated with Risk of Anxiety in Humans

(A–C) Regional association ($-\log_{10}$ of the p value) plot for SNPs in the *SIRT1* gene region for any anxiety disorder (A), panic disorder (B), and major depressive disorder (C). Physical positions for each of the 14 SNPs tested, the *SIRT1* gene (arrows depicting direction of transcription), and *HERC4*, a neighboring gene, are shown (International HapMap Consortium, 2003, release #28). The SNP with the best association is denoted by a red square. The best association for any anxiety disorder was observed with SNP rs10997870, for panic disorder with SNP rs12778366, and for major depressive disorder with SNP rs12413112.

(D) A summary of the associations between SNPs in *SIRT1* and anxiety disorders and depression in humans, in three independent population-based cohorts of people (from Europe, USA, and Japan; Kishi et al., 2010; also see Figure S5). The trait, SNP ID and position, minor allele frequency (MAF), allele frequency in cases, allele frequency in controls, t-statistic coefficient (Stat), nominal p value (p), Bonferroni-corrected p value (Bonf. P), and permutation-based corrected p value (Permut. P, N = 10,000) are presented. PsyCoLaus cases from Lausanne (Preisig et al., 2009) were white Caucasians who met the criteria for any of the following: agoraphobia (n = 122), generalized anxiety disorder (n = 62), panic disorder (n = 92), social phobia (n = 342), post-traumatic stress disorder (n = 133), or depression (n = 1255). Controls were Caucasians who did not meet criteria for any of these disorders. Cases for the Virginia Study (Hettema et al., 2008) were adults who met criteria for panic disorder (n = 117). Note that rs10997870 is in strong LD with rs10997875 in Europeans and Japanese populations (E).

(E) LD evaluation of common SNPs in *SIRT1*. ATG denotes the *SIRT1* translation start codon, vertical bars represent *SIRT1* exons, and TAG denotes the stop codon. Shaded diamonds depicting LD between markers (black shading represents complete LD, and white shading, no LD) are based on the R^2 measure, where a value of “93” corresponds to $R^2 = 0.93$. Note, a number of SNPs showing nominal association ($p \leq 0.05$) with mood disorders in this study are in moderate to strong LD.

(F) We duplicated two of the most frequent mutations in the N terminus of *SIRT1* (S14P and P37L, see Figures S5A, S5C, and S5D) in our overexpression plasmid. SDS-PAGE blot is shown to demonstrate overexpression of mutant human *SIRT1* proteins in N2A cells.

food is scarce, the population of the species of interest drops, and predation is high (Figure 7F, red shaded area). Food abundance suppresses SIRT1 activity in many regions of the brain (Chen et al., 2008), including the amygdala (Figures 7G and 7H), resulting in increased exploratory drive and reduced anxiety. This strategy makes sense because a time of food abundance and low predation would be propitious for radiation of the species. Alternatively, food scarcity will activate SIRT1 (Figures 7G and 7H), increasing anxiety and decreasing exploratory drive. This strategy also makes sense because a time of food scarcity and high predation would behoove high vigilance, as discussed for CR above. In this regard, it is fascinating that nursing mothers put on calorie restriction have been reported to yield pups that have a suppressed exploratory drive no matter how they are fed later in life (Franková and Barnes, 1968). In summary, SIRT1 may trigger psychological changes to complement its known effects on stress-response pathways to ensure longer survival in adverse conditions.

Conclusion

Here we describe a mechanism by which SIRT1 in the brain directly regulates mood and behavior by deacetylating the NHLH2 transcription factor that activates MAO-A transcription. In addition, unbiased human-based population studies revealed that both common and rare variants in *SIRT1* are associated with anxiety and other psychiatric disorders. These data provide evidence for the role of SIRT1 in modulating mood and behavior, which will help guide the development of brain-permeable SIRT1 drugs for psychiatric and neurodegenerative diseases. Additionally, we describe a theoretical evolutionary model for adaptation of a species to food availability and predation that helps rationalize the link between SIRT1 and anxiety. Foraging-predation risk trades-offs strongly influence evolution and define ecosystems, and nutrient-sensing enzymes such as SIRT1 may directly orchestrate these events by exerting psychological, as well as physiological, effects.

EXPERIMENTAL PROCEDURES

Mice and Behavioral Tests

All procedures were performed according to guidelines and under supervision of the Committee for Animal Care (CAC) of Massachusetts Institute of Technology. For all tests, we used 3-month-old animals. All mutants were compared to corresponding WT littermates. Males were single housed and handled daily for 2 weeks prior to the test to eliminate the influence of hierarchy on their behavior and to minimize anxiety due to human handling. In all tests, animal behavior was filmed and analyzed later with the help of custom-written software.

Where applicable, intraperitoneal injections of 20 mg/kg of body weight of phenelzine or fluoxetine were given to each mouse every other day for 2 weeks and 1 hour prior to each behavior test.

RT-PCR

cDNA was prepared using RetroScript (Ambion) as per manufacturer's instructions. Abundance of specific RNA in samples was normalized to ribosomal

protein rpl19. RT-PCR was performed using the Roche 480 system, with the following primers: MAO-A-F: GCCCAGTATCACAGGCCAC; MAO-A-R: CGGGCTTCCAGAACCAAGA; rpl19-F: ATGAGTATGCTCAGGCTACAGA; rpl19-R: GCATTGGCGATTTCATTGGTC.

LC-MS/MS

After in-gel digestion of samples with trypsin, they were analyzed using reversed phase microcapillary LC-MS/MS. It was performed using an EASY-nLC nanoflow HPLC (Proxeon Biosciences) with a 75 μm id \times 15 cm C_{18} column connected to a hybrid LTQ linear ion trap-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) in data-dependent acquisition and positive ion mode at 300 nL/min followed by protein database searching using Sequest.

Plasmids

MAO-A-luciferase reporter plasmid was a generous gift from U. Albrecht and has been described previously (Hampp et al., 2008). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with Renilla as the reference. The plasmid encoding human NHLH2 was purchased from OriGene (TrueORF collection). Detailed methods can be found in Extended Experimental Procedures.

Genetic Analysis

Genotyping of SIRT1 tag-SNPs was conducted by KBioscience (<http://www.kbioscience.co.uk/>), and DNA sequencing was performed by Agencourt Biosciences using capillary sequencing on genomic DNA samples from a subset of the PsyCoLaus population-based cohort from Lausanne, Switzerland.

Statistical Analysis

Unless otherwise noted, results are expressed as mean \pm standard error of the mean (SEM). Differences between two groups ("cases" and "controls") were assessed with unpaired two-tailed t tests. The Extended Experimental Procedures contain detailed statistical analysis of all experiments using t test, u test, Kruskal-Wallis, and ANOVA where applicable. For the PsyCoLaus human association studies, we used stringent quality-control criteria, requiring Hardy-Weinberg p values $>10^{-7}$, $<30\%$ missing data per SNP, $<10\%$ missing data per individual, and an SNP minor allele frequency >0.01 . In each analysis, the regression was carried out including age, sex, and the first two genotype principal components as covariates. Analysis was carried out in PLINK (Clarke et al., 2011) using a logistic regression. The permutation tests were carried out using 10,000 permutations in PLINK.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2011.10.054.

ACKNOWLEDGMENTS

We would like to acknowledge Vincent Mooser, Federica Tozzi, Kijung Song, James Ellis, Enrique Castelao, and Carol Prescott for their contribution to the provision, analysis, and interpretation of human genetic data; and Xuemei Yang for help with mass spectrometry. This work was supported by the fellowship from Leukemia and Lymphoma Society (5089-09) to S.L. and grants from the NIH and a gift from the Glenn Foundation for Medical Research to L.G. The PsyCoLaus study was supported by grants from the Swiss National Science Foundation (#3200B0-105993, #3200B0-118308, #33CSC0-122661) and from GlaxoSmithKline. S.B. was supported by the Swiss National Science Foundation (#31003A_130691), the Swiss Institute of Bioinformatics, and the

(G) S14P and P37L SIRT1 mutant proteins had enhanced ability to activate MAO-A (as measured by luciferase reporter) than similarly expressed WT protein. Such an enhanced activity of SIRT1 in the brains of these subjects might increase their susceptibility to mood disorders via the mechanism that we discuss in this paper (also see Figures S5C and S5D). Box-plots represent median, 25th, and 75th percentiles of data. Whiskers present 5th and 95th percentiles of data, with actual data points on the right. p values are calculated using t test.

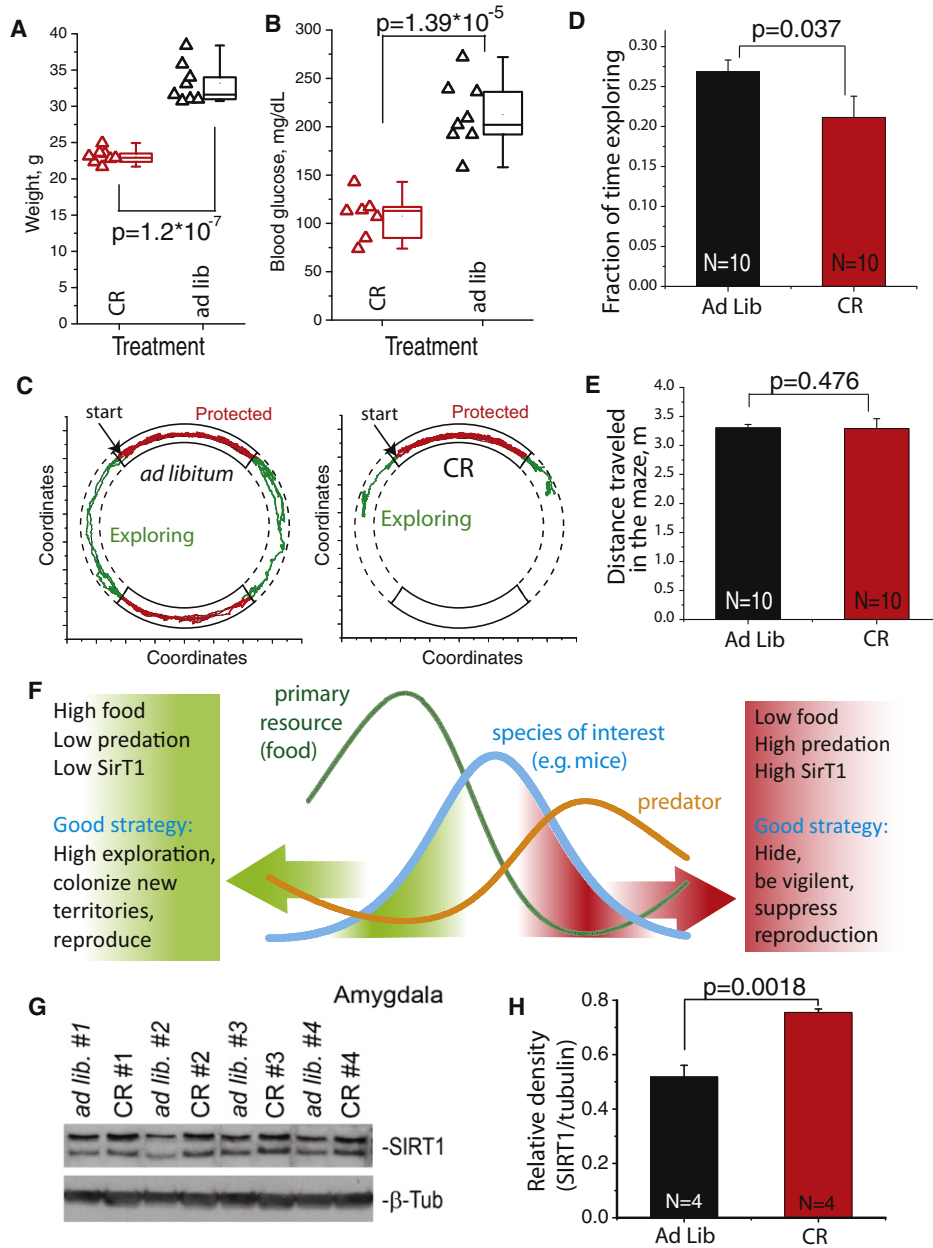


Figure 7. Evolutionary Theory Explains Advantages of Anxiety Control by Food Availability

(A and B) Calorie-restricted (CR) mice have lower body weight (A) and lower blood glucose (B). Box-plots represent median, 25th, and 75th percentiles of data. Whiskers present 5th and 95th percentiles of data, with actual data points on the left. p values are calculated using t test.

(C and D) CR mice are more anxious and explore less in the zero-maze than their counterparts fed ad libitum diet. Typical tracking diagrams (C) are shown for CR and ad libitum mouse. Mice were placed into zero-maze apparatus (see Figure 1B) always in the same position “start.” Behavior of animals was filmed, and their movement was digitally tracked (Image-J, NIH). Upper and lower quadrants on the track-graph represent areas in the zero-maze that were protected by walls (red dots). Side quadrants of the graph represent open arms of the zero-maze (green dots). Fraction of time spent exploring (fraction of time spent on the open arms of the maze) is quantified and presented (D). Mean \pm SEM is shown.

(E) CR and ad libitum mice have similar activity while inside the zero-maze. We find that CR mice travel a similar distance to that of ad libitum animals (E), yet they explore less (D). Data presented as mean \pm SEM. p values are calculated using t test.

(F) Evolutionary rationale for mood regulation by SIRT1 (also see Figure S6). In the three-species food chain, the population sizes will oscillate as shown. In the left part of the cycle (green shaded area), food abundance is high (rendering SIRT1 activity low) and predation is low, which suggest that low anxiety and high exploration is the best strategy. In the right part (red shaded area), food is scarce (rendering SIRT1 activity high) and predation is high, suggesting that minimal exploration with maximum vigilance is the best strategy.

(G) Animals subjected to CR have more SIRT1 in amygdala obtained by microdissection. SDS-PAGE for SIRT1 is presented, with tubulin as loading control.

(H) Quantification of data presented in (G) is shown using the densitometry function of Image-J software (NIH). Mean \pm SEM is shown.

European Framework Project 6. We are grateful to Mid-Atlantic Twin Registry (MATR) for the ascertainment of VATSPSUD subjects for the replication study. The MATR was supported by grant UL1RR031990 from the National Center for Research Resources. J.R. works for GlaxoSmithKline. L.G. consults for Sirtris (GSK company).

Received: April 28, 2011
 Revised: August 25, 2011
 Accepted: October 12, 2011
 Published online: December 8, 2011

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