Regulation of Neuronal Genomic Integrity through Histone Deacetylase Cooperativity

By
Matthew Milnes Dobbin
B.S., The University of Vermont (UVM), (2005)
Submitted to the Department of Brain and Cognitive Sciences
In Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy in the field of Neuroscience
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Abstract
While the mechanisms preserving genomic integrity are well defined in proliferating cells, corresponding pathways in postmitotic neurons remain poorly understood. In this report, I characterize the functions of two lysine deacetylases, SIRT1 and HDAC1, in the neuronal response to DNA double strand breaks (DSBs). Both SIRT1 and HDAC1 were previously shown to promote neuronal survival in a mouse model of neurodegeneration in which the appearance of DSBs precedes other neurotoxic symptoms. Here I show for the first time the recruitment of both SIRT1 and HDAC1 to sites of DNA DSBs in neurons, where they work cooperatively to coordinate DSB signaling and DNA repair. SIRT1 physically binds HDAC1 and this interaction is strengthened upon DNA damage. I demonstrate that SIRT1 deacetylates HDAC1 at a critical lysine residue, K432, and stimulates its enzymatic activity. Moreover, HDAC1 mutants that mimic a constitutively acetylated state render neurons more susceptible to DNA damaging agents, and pharmacological SIRT1 activators that promote HDAC1 deacetylation also mitigate neuronal loss in a mouse model of neurodegeneration. I propose that the interaction between SIRT1 and HDAC1 constitutes an essential step in the DNA damage response that could be exploited to enhance neuronal survival in various neurodegenerative diseases.

Thesis Supervisor: Li-Huei Tsai

Title: Picower Professor of Neuroscience
Department of Brain and Cognitive Sciences
Director, The Picower Institute for Learning and Memory
Table of Contents

CHAPTER 1: Introduction
  1.1 Background.................................................................................................6
  1.2 Organization of Thesis...............................................................................24

CHAPTER 2: SIRT1 and HDAC1 are Functional Interaction Partners
  2.1 Summary.......................................................................................................27
  2.2 Introduction...................................................................................................28
  2.3 Experimental Procedures.............................................................................30
  2.4 Results...........................................................................................................35
  2.5 Discussion......................................................................................................50

CHAPTER 3: SIRT1 and HDAC1 are Apical Components of the Neuronal DNA Damage Signaling and Repair Pathway, and their Cooperativity is Required for Maintenance of Genomic Integrity
  3.1 Summary.......................................................................................................55
  3.2 Introduction...................................................................................................57
  3.3 Experimental Procedures.............................................................................59
  3.4 Results...........................................................................................................62
  3.5 Discussion......................................................................................................101

CHAPTER 4: Discussion
  4.1 Introduction................................................................................................105
  4.2 References..................................................................................................113

ADDENDUM: Dobbin et al 2013, Nature Neuroscience Complete Manuscript.................................................................121
CHAPTER 1: Introduction
DNA comprises the fundamental functional base structure through which genetic information is stored and transmitted across cellular generations and from parent to offspring. Given that abnormalities at even the single nucleotide resolution can readily lead to diseases and disorders that can threaten the survival of an organism, complex molecular mechanisms have evolved to ensure the fidelity of DNA. The importance of these mechanisms is particularly evident in the nervous system: compromised DNA repair capabilities manifest profoundly, resulting in a number of diseases including neurodegenerative syndromes, microcephaly, brain tumors, and aging.

Within the nervous system, the origin of genotoxic insult arises from largely endogenous sources. Given the high metabolic load typical of neurons when compared to other cell types, free radicals and oxidative stress have been shown to be the primary contributors of DNA damage burden. Furthermore, free radicals and oxidative stress can produce multiple different types DNA damage, each requiring the activation of repair mechanisms dedicated to the remediation of each unique form of damage, specifically. Additionally, normal neural functioning can invoke the DNA damage response, as glutamatergic signaling and receptor activation can lead to the phosphorylation of histone H2AX (YH2AX), which is considered one of the early steps in the DNA damage response and repair machinery.
Table 1. DNA Repair Disorders Affecting the Human Nervous System

<table>
<thead>
<tr>
<th>Disease/Syndrome</th>
<th>Gene</th>
<th>Clinical Presentation</th>
<th>Mouse Models</th>
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<tbody>
<tr>
<td><strong>Nucleotide Excision Repair</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Xeroderma Pigmentosa (XP)</td>
<td>XPA-G</td>
<td>microcephaly, degeneration, mental retardation, cerebellar ataxia, extreme UV sensitivity</td>
<td></td>
</tr>
<tr>
<td>Cockayne Syndrome (CS)</td>
<td>CSA/B, ERCC6/8, XPB/D/G</td>
<td>microcephaly, degeneration, loss of myelination, progeria, deafness, UV sensitivity</td>
<td></td>
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<tr>
<td>Trichothiodystrophy (TTP)</td>
<td>TTD-A</td>
<td>microcephaly, mental retardation, deafness, ataxia, UV sensitivity</td>
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<tr>
<td><strong>Single Strand Break Repair</strong></td>
<td></td>
<td></td>
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<tr>
<td>Ataxia and Oculomotor Apraxia-1 (AOA1)</td>
<td>APTX</td>
<td>ataxia, oculomotor apraxia, cognitive impairment, cerebellar atrophy/degeneration</td>
<td></td>
</tr>
<tr>
<td>Spinocerebellar Ataxia and Axonal Neuropathy (SCAN1)</td>
<td>TDP1</td>
<td>cerebellar atrophy/degeneration</td>
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<tr>
<td><strong>Double Strand Break Repair</strong></td>
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<tr>
<td>Nijmegen Breakage Syndrome (NBS)</td>
<td>NBS1</td>
<td>microcephaly, extreme cancer predisposition</td>
<td></td>
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<tr>
<td>Ataxia Telangiectasia (A-T)</td>
<td>ATM</td>
<td>ataxia, cerebellar atrophy/degeneration, oculomotor apraxia, extreme cancer predisposition</td>
<td></td>
</tr>
<tr>
<td>Seckel Syndrome (ATR)</td>
<td>ATR</td>
<td>microcephaly, dwarfism</td>
<td></td>
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<td>Ataxia Telangiectasia-Like Disease (ATLD)</td>
<td>MRE11</td>
<td>ataxia, oculomotor apraxia</td>
<td></td>
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<td>LIG4 Syndrome</td>
<td>LIG4</td>
<td>microcephaly</td>
<td></td>
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<tr>
<td>XLF Syndrome</td>
<td>XLF/NHEJ1</td>
<td>microcephaly, general growth retardation</td>
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</table>
Various different forms of DNA damage exist for which there are distinct pathways that have evolved to deal with them. These different forms consist of nucleotide/base excision repair (BER), single strand break repair (SSBR) and double strand break repair (DSBR). Deficiencies in any of these pathways can lead to human pathologies and neurological disease.

Nucleotide excision repair deals largely with resolving damage originates from ultraviolet (UV) radiation which produces lesions that distort the 3-dimensional structure of DNA. These distortions, referred to as DNA adducts, consist primarily of thymine dimers and 6'-4' photoproducts. Nucleotide excision repair can be further broken down into two distinct subclassifications: global genomic NER (GGNER) and transcription-coupled NER (TCNER). GGNER and TCNER subpathways differ in the ways in which damage is recognized/sensed, but share the mechanism through which damaged DNA is excised, repaired, and ultimately ligated. Irrespective of how damage is initially recognized, rectification of damage via NER involves the removal of a short tract of nucleotides from the strand containing the lesion (damaged DNA). Sequence from the undamaged complementary strand is subsequently used as a template for synthesis by DNA polymerase, after which the newly synthesized sequence is ligated back into the excised strand by DNA ligase IV (LIG4), reconstituting the double-stranded structure and completing NER.

Figure 1. The global genomic nucleotide excision repair pathway (illustration adapted from Ciccia and Elledge, 2010)
GGNER is responsible for constantly scanning the genome for damage occurring in both actively transcribed and transcriptionally inactive genes. Detection of lesions via the NER pathway is not dependent upon transcription, and is carried out by damage sensing complexes comprised of XPC-Rad23B dimers. XPC is a DNA-binding protein and is thought to initially sense damage by binding to unpaired nucleotides that become exposed as a result of conformational changes in the DNA backbone incurred by formation of either thymidine dimers or 6'-4' photoproducts. While XPC alone does possess minimal ability to facilitate repair, its activity is maximized when bound to Rad23B ubiquitinase which is thought to inhibit proteasomal degradation of XPC.

Figure 2. Types of DNA damage and repair (illustration adapted from Ciccia and Elledge, 2010).
While XPC is the best characterized member of the XP family of genes, mutations and abnormalities in several members of the XP group have been linked to the disease for which they are namesake, xeroderma pigmentosum. Xeroderma pigmentosum is an autosomal recessive genetic disease with a population frequency of 1 in every 250,000 individuals across all races and ethnic groups, with a survivability rate of less than 40% above the age of 20 years old. The primary clinical manifestation of xeroderma pigmentosum is extreme sensitivity to UV wavelength (100nm-400nm) radiation, producing characteristic discoloration of skin pigmentation in those affected, who have over a 1,000 fold increased likelihood of developing skin cancer in cells exposed to UV light. Within the context of the nervous system, individuals affected by XP display variably severe microcephaly (a head circumference of more than two standard deviations below the mean for age and gender), mental retardation, cerebellar degeneration, and cerebellar ataxia. Current understanding regarding the neurological causes of XP remain poorly understood, although emerging evidence suggest a strong linkage to a developmental inability to programmatically eliminate (apoptose) cells destined to neurons/nervous tissue, resulting from XPC being unable to scan for and sense lesioned genomic DNA.

As its name implies, transcription-coupled nucleotide excision repair (TCNER) is inherently tied to the transcriptional machinery, and thusly occurs in
transcriptionally active genes. Lesions occurring within actively transcribed sequences is prioritized over those occurring in transcriptionally inactive genomic DNA, and is typically repaired faster. As previously mentioned, GGNER and TCNER differ only in the way in which they initially sense and detect damage. Both pathways converge for the incision, ligation, and repair steps of the overarching NER pathway. Unlike GGNER, TCNER requires neither XPC nor Rad23B to scan for and detect conformational changes in DNA structure resulting from lesions. In actively transcribed regions, RNA polymerase II α (RNAPII) unable to progress along synthesized template directly acts as a signal indicating damage, as dimerized pyrimidines block and stall RNAPII. Following initiation of TCNER repair upon stall of RNAPII, the polymerase complex is then moved backwards to facilitate access to the lesion for downstream repair components. It has been shown that CSB and XPG recognize and directly bind damaged DNA specifically in actively transcribed regions much in the same way that XPC and Rad23B accomplished the same task in GGNER.

Figure 2. The transcription-coupled nucleotide repair pathway (Illustration adapted from Ciccia and Elledge, 2010)
Two diseases have been linked to defects in the TCNER pathway: trichothiodystrophy and Cockayne Syndrome.

Trichothiodystrophy (TTD) is an autosomal recessive disorder and is sometimes referred to as Amish brittle hair brain syndrome, as its frequency is high within the Amish diaspora where marriage and reproduction outside of the community is discouraged. Individuals affected by TTD display characteristically brittle hair, ichthyosis, short stature, and decreased fertility. Neurological implications of TTD include microcephaly and mental retardation.

Another genetic disorder involving dysfunction of TCNER is Cockayne Syndrome. It is an extremely rare autosomal recessive disorder with severe neurological implications. Cockayne Syndrome results from mutation in the CSB, which is responsible for detecting structural changes in DNA resulting from lesion formation and is RNAPII-independent. Those afflicted by Cockayne Syndrome do not typically exhibit symptoms until after the age of 2 after which severe and rapid neurological degeneration occurs, ultimately resulting in death within the first two decades of life. A general “aged” look is typical following onset, in addition to loss of hearing, vision, nystagmus, and UV hypersensitivity.

Unlike DNA damage that invokes nucleotide excision repair machinery, the single-strand break DNA repair pathway is induced by physical breaks in one of the two DNA strands comprising the DNA double-stranded helix. One of the most common sources of single-strand DNA breaks arises from reactive oxygen species, most commonly H₂O₂ that is generated as a byproduct of the citric acid cycle, and represents an endogenous source of damage that is constantly generated under normal physiological conditions. Single-strand breaks have been shown to occur at a frequency of three orders of magnitude more than double-strand breaks in cells of non-neuronal origin, and likely occur at an even significantly higher frequency in neurons, given their high metabolic load. Successfully detecting and repairing damage therefore represents a critically vital cellular function for neurons whose total population is fixed at birth. Another source of endogenous single-strand DNA breaks arise from erroneous or abortive DNA topoisomerase activity. Topoisomerases are a family of enzymes
that function as endonucleases: they cleave the phosphate backbone of double-helical DNA and facilitate the unwinding and decondensing supercoiled DNA. Higher-order DNA structures are extremely important functionally, as this topology serves to both inhibit errant and undesirable transcription and also to protect DNA from damage by physically sequestering (internalizing) genetic material into highly condensed coil structures physically analogous to tightly wound rubber bands. However, in order for processes such as transcription and replication to occur, supercoiled DNA must first be decondensed to return DNA to a physically permissive state that is readily accessible to large macromolecular DNA/RNA polymerase complexes. Topoisomerases accomplish this cutting the phosphodiester DNA backbone thereby relieving the considerable torsional stress DNA assumes as it is packaged into a supercoiled state. Only then can processes requiring accessibility to "naked" DNA occur. While cleavage "nicks" are typically transient and are rapidly resealed by the very topoisomerase that created them, RNA and DNA polymerases that follow in close spatial proximity can collide with and stall topoisomerase on the strand that it is actively cleaving. As the enzymatic reaction cycle involves covalent linkage with the phosphodiester backbone, a stalled topoisomerase will remain otherwise irreversibly stuck to the 3'-terminus of the DNA strand it was in the process of cleaving until it is actively removed. This effectively results in the formation of single stranded break.

DNA topoisomerase I (TOPI) has garnered significant attention recently, as it has been recently linked to the disease spinocerebellar ataxia with axonal neuropathy 1 (SCAN1). SCAN1 is an autosomal recessive genetic disorder affecting every 1 in 500,000 Americans. Age of onset is typically late childhood (13-15 years of age), and characterized by slowly progressive cerebellar ataxia. As the disease progresses, nociception and tactile sensation decrease and patients steadily lose physical control despite retaining full cognitive ability and patients can live relatively long lives.

Another disease having defects in single-strand break repair as its cause is ataxia and oculomotor apraxia (AOA1). It is an autosomal recessive genetic
disorder with a mean onset of ~4 years of age and is characterized by slowly progressive cerebellar ataxia followed by oculomotor apraxia (loss of voluntary eye control), and severe axonal neuropathy. Complete loss of ambulation and quadriplegia is typical experienced ~10 years following disease onset. Despite this, patients are capable living long lives if provided with therapy and daily assistance as the disease progresses. Although the cause of AOA1 has been linked to the gene APTX, its role still remains unclear.

DNA double-strand breaks (DSBs) are breakages in both strands of the DNA double helix. Unlike single-strand breaks, DSBs pose a unique threat to cells in that unrepaired DSBs can lead to chromosomal and genomic rearrangements. It is estimated that DSBs occur at a rate of 10 breaks per cell, per day. This obviously represents a significant threat to both cellular stability and survival. While a very specific subset of DSBs are formed as a result of normal physiological processes, such as during recombination that occurs in lymphocytes undergoing generation of immunogenicity via V/D/J recombination, all other DSBs formed can be considered pathological. To ensure that DSBs are repaired properly, cells rely upon two distinct pathways: homologous recombination and non-homologous end-junction. Reactive oxygen species (ROS) are again a major source of DSBs. While undergoing normal cellular respiration ~0.1-1% of oxygen is converted to superoxide (O$_2^-$) in the mitochondria, which is then converted to hydroxyl free radicals by either superoxide dismutase 1 or 2 (SOD1/2). These hydroxyl free radicals are then capable of going on to cause single-stranded breaks, as previously mentioned. About $10^9$ free radicals are produced in the body per cell, per hour, and if two lesions form in close proximity to each on antiparallel strands, a DSB will occur.

Homologous recombination differs from non-homologous end junction in that it relies upon the availability of either a sister chromatid (available only in G2 phase following DNA replication) or a sister chromosome to function as a template for synthesis to replace a short segment of the damaged DNA that must be excised. The requirement for availability of sister chromosome to function as template means that the sister chromosome must be both mobile and also in
close proximity to the DSB, which must also be mobile. This represents a
significant barrier to repair, as it highly likely that the sister chromosome will
topologically constrained and packed in higher order chromatin structures. As
such, homologous recombination is thought to be restricted almost entirely to
cells that are actively dividing and actively mitotic as chromatin is already in a
decondensed state in actively dividing cells and they can furthermore rely upon
the availability of sister chromatid to function as a template for repair.

Despite being largely restricted to mitotically active cells, genetic defects
in homologous recombination repair pathways have been linked to diseases
affecting the nervous system. Ataxia telangiectasia-like disorder (ATLD) has
been mapped to a homozygous or compound heterozygous mutation in the gene
MRE11A on chromosome 11q21. MRE11 is a nuclease whose role is to excise
short strands of sequence immediately 3′ to adjacent double-strand break sites,
thereby revealing a short strand referred to as a microhomology domain that will
serve as a site into which the newly synthesized and undamaged sequence can
be ligated into. ATLD is an autosomal recessive disorder that is characterized
by ataxia resulting from progressive cerebellar degeneration, oculomotor apraxia,
and extreme UV/radiosensitivity. As it is a rare disorder, reliable statistics
concerning lifespan and population frequency are not currently available. The
fact that a defect in a homologous recombination repair pathway protein can lead
to a disease that largely affects the nervous system. It is currently theorized that
because HR is defective in individuals affected ATLD, even though DNA DSBs
are occurring in cells destined to become neurons/nervous tissue, breaks are not
repaired and mutations/abnormal structures eventually become incorporated into
the genome within genes critical to neuronal function.

NBS1 is a binding partner of MRE11 along with Rad50. Together they
comprise the M/R/N complex and this protein complex is solely responsible for
responding to pathological DNA DSBs through the homologous recombination
pathway. Nijmegen breakage syndrome is the disorder for which NBS1 is
named after, and mutation in NBS1 is the underlying genetic cause of the
disease. It is an autosomal recessive disorder characterized by microcephaly, growth retardation, and predisposition to cancer.

Perhaps the most well known disease affecting the nervous system with a defect in a DNA damage repair pathway is ataxia telangiectasia (AT). Ataxia telangiectasia is an autosomal recessive genetic disorder that affects between 1-40,000 and 1-100,000 people worldwide. Age of onset is between 3-5 years old and is characterized by a number of neurological features, the most notable of which is ataxia and secondarily oculomotor apraxia and postural instability. Cerebellar degeneration progresses throughout childhood and affected individuals can be expected to typically live into early adulthood. The genetic basis for ataxia telangiectasia is a mutation in the gene ataxia telangiectasia-mutated (ATM), which was first cloned in 1995 and is located in 11q22.3. As AT is an autosomal recessive disorder, affected patients receive one mutated copy from each parent. Prenatal screening is therefore recommended for parents in which one member is known to carry a mutated ATM allele. The protein product encoded by ATM is a serine/threonine kinase that is activated by DNA double-strand breaks, and is a member of the phosphatidylinositol kinase related-kinase (PIKK) family of proteins. The primary target of ATM is the histone variant H2AX. In response to double-strand breaks, ATM is activated and then goes on to immediately phosphorylate serine 139 of H2AX, amongst many other targets. H2AX phosphorylated at serine 139 is referred to as γH2AX, and γH2AX forms immediately adjacent to double-strand break sites and subsequently spreads for several megabase pairs both upstream and downstream of break sites. This is an extremely important step in the repair process and is thought to comprise one of the most upstream and critical events that is shared by virtually all DNA repair pathways. It is therefore considered to be an apical step in the DNA damage repair signal transduction pathway, and is thought to serve as both a molecular flag/signal indicating where damage has occurred and also as a platform onto which downstream complexes involved in repair pathways.

Ataxia telangiectasia-related (ATR) is a gene very closely related to ATM. The protein product encoded by ataxia telangiectasia-related is also a
serine/threonine kinase and member of the PIKK family. Unlike ATM, which is responsible for the initial detection of DNA double-strand breaks, ATR is activated by the persistent presence of single-strand breaks. ATR also phosphorylates H2AX at serine 139, but only in the presence of single-stranded breaks. Interestingly, ATM and ATR are interdependent as propagation of the γH2AX signal adjacent to the break after initial detection, regardless of whether it is single- or double-stranded, relies upon both ATM and ATR. ATM and ATR exist in a larger protein complex, and both are required for upstream and downstream spread of γH2AX signal flanking a break. Mutation in ATR (located on chromosome 3q22-q24) has been shown to be responsible for Seckel Syndrome. Seckel Syndrome, also known as bird-headed dwarfism, is an extremely rare autosomal recessive disorder characterized by intrauterine growth retardation, severe microcephaly, and intellectual disability (more than 50% of patients have an IQ of 50 or below).

Another disease associated with a deficiency in DNA double-strand break repair is LIG4 Syndrome. It is named for the gene in which mutation results in the disease: ligase IV (LIG4). As its name implies, ligase IV is a DNA ligase that is a component in the non-homologous end joining repair pathway. It is ultimately responsible for one of the last steps in the NHEJ repair cycle: the ultimate rejoining of repaired DNA double-strands breaks to reconstitute the DNA duplex. LIG4 Syndrome is an extremely rare autosomal recessive disorder caused by a homozygous or compound heterozygous mutation in LIG4. LIG4 syndrome is characterized by severe microcephaly, developmental delay, growth retardation and ionizing radiation sensitivity.

**Origins and Sources of DNA damage**

As previously mentioned, DNA damage can arise from a multitude of sources, both endogenous and exogenous. Estimates of the frequency of types of DNA damage can vary greatly between cell types and species. Endogenously occurring forms of DNA damage includes oxidative damage, depurinations, depyrimidations, single-strand breaks, double-strand breaks, O6-methylguanines, and cytosine deamination.
Oxidative Damage
humans (per cell per day): ~10,000-11,500
~2,800 (8-oxodG)
rats (per cell per day): ~75,000-100,000
mouse (per cell per day): ~34,000 (8-oxodG)

Depurinations
mammalian cells (per cell per day): ~2,000-12,000

Depyrimidations
mammalian cells (per cell per day): ~700

Single-strand breaks
mammalian cells (per cell per day): ~55,000

Double-strand breaks
mammalian cells (per cell per day): ~1,000

O6-methylguanines
mammalian cells (per cell per day): ~3,000

Cytosine deamination
mammalian cells (per cell per day): ~192

Oxidative damage is caused by reactive oxygen species (ROS), of which there are a number of different types. Of the various different types of reactive oxygen species, hydroxyl radical (\(\cdot\)OH) is the most highly reactive. Hydroxyl radical reacts with DNA by addition to double bonds of nucleotide base pairs and by abstraction of a hydrogen atom from the methyl group of thymine and each of the C-H bonds of 2'-deoxyribose. Addition to the C5- C6- double bond of pyrimidines leads to formation of adducts that vary in terms of their redox properties, with C5-OH adduct radicals being reducing and C6-OH adduct radicals being oxidizing. Another major source of oxidative damage is the process of oxidative deamination. DNA damage formed through oxidative deamination is largely restricted to the liver and kidneys and can lead to the formation of both H₂O₂ (hydrogen peroxide) and ammonia. As has already been mentioned, reactive oxygen species are formed primarily as a byproduct of the
citric acid cycle and normal cellular respiration. As neurons have metabolic load that is considerably higher than any other cell type, it is of the utmost importance that they be able to effectively repair damage incurred by reactive oxygen species.

The other primary source of endogenous damage comes from aberrant or abortive activity of nuclear enzymes that directly act upon DNA for various cellular functions. These can include DNA polymerases, RNA polymerases, and topoisomerases. Should any of these enzymes stall in the middle of carrying out their respective molecular processes, damage repair pathways will be invoked.

In addition to the numerous forms of DNA damage that originate innately as a result of normal physiological cellular processes, cells are constantly exposed to environmental insults that can also lead formation harmful damage lesions. Perhaps the most prominent source of environmental damage is from ionizing radiation. Ionizing radiation includes gamma radiation, and x-ray radiation. At sea level ~300 million ionizing radiation particles per hour pass through each person. As these particles pass through the body, they generate free radicals by interacting with water molecules, and the free radicals can then go on to create both single- and double-stranded breaks. Furthermore, ionizing radiation can directly result in the physical creation of DNA double strand breaks. Over half of all ionizing radiation encountered by cells arises from off of the earth (the sun), while the other half arises from the radioactive decay of elements. Additionally, ultraviolet radiation can also induce DNA damage lesions. While UV-B wavelength light (290nm-320nm) directly causes damage by crosslinking adjacent pyrimidine dimers (thymine and cytosine bases), UV-A wavelength light (320nm-400nm) can indirectly result in creation of DNA damage as light of this wavelength leads to generation of free radicals and reactive oxygen species.

Signals and Substrates for Repair
Perhaps the most robust signal that can induce DNA damage repair and response pathways is a stalled replication fork. A single-stranded break occurring on either the leading strand or lagging strand can invoke DNA repair processes. If the break is on the leading strand, then an open-ended double-strand break will result, and DNA synthesis can be restarted by break-induced replication. Leading strand synthesis can also be blocked, leading to uncoupling of leading strand and lagging strand synthesis. Such would be the case if a replisome (DNA replication macromolecular complex) encountered a pyrimidine dimer created by UV-B damage on the leading strand. This causes reversal of the replication fork, leading to formation of a Holiday Junction, or chickenfoot DNA structure. Replication can restart once the Holiday Junction is resolved back into a replication fork or the Holiday Junction is completely cleaved off and break-induced replication is activated. Damage occurring on the lagging strand by nature creates a single-stranded DNA break. Unlike pyrimidine dimers occurring on the leading strand, a pyrimidine dimer on the lagging strand will not halt progression of the replisome and synthesis of the leading strand. Errors in lagging strand synthesis are fixed downstream by either trans-lesion synthesis or error-free homologous recombination, which relies upon the availability of a sister chromatid for repair.

Apurinic/apyrimidinic sites are also common lesions that can invoke the DNA damage response. They can form either spontaneously, but can also form in the process of normal repair itself. An apurinic/apyrimidinic site is a position in the DNA sequence in which a base is literally missing. They are most commonly formed by attack from radical hydroxyls on the deoxyribose moiety, causing release of free bases from the DNA backbone. Endogenous reactive oxygen species can cause up to 50,000-200,000 apurinic/apyrimidinic sites per cell per day.

One of the most commonly encountered forms of DNA base damage is 7,8-dihydro-8-oxoguanine (8-oxoG) and 8-hydroxy-2-deoxyguanosine (OHdG). Both 8-oxoG and OHdG are created by hemolytic cleavage by H₂O₂.
Interestingly, specific sites and sequences seem to preferentially accumulate this type of damage.

Of the various types of DNA damage, double-strand breaks are perhaps the most dangerous. Double-strand breaks can form through a variety of different mechanisms. The primary source of endogenous double-strand breaks is when DNA replication forks encounter unrepaired lesions, triggering replication fork collapse. In fact, the frequency of this is so high that in-vivo leading and lagging strand is considered to be discontinuous by some, not semi-discontinuous.

**Sensing and Recognition of DNA damage**

Much in the same way that an enzyme or receptor recognizes its cognate substrate, the simplest way in which a DNA damage lesion is recognized is by a cognate protein that specifically targets and binds the 3-dimensional lesion structure/conformation. Specific examples of proteins that directly recognize damage includes DNA photolyases and glycosylases. Photolyases specifically recognize photoinduced pyrimidine dimers through direct interaction with the DNA backbone that is in a distorted state resulting from change in conformation resulting from the pyrimidine dimer. Upon binding the dimer, photolyase weakens the bonds between both the pyrimidine dimer and complimentary purines which allows for rotation of the dimer around the phosphodiester backbone. This flipping mechanism transposes the crosslinked dimer into the substrate pocket of the photolyase where the crosslink can then be hydrolyzed and the damage repaired.

Often times damage is not directly recognized and repaired by one single protein as photolyase does. Recognition of damage often involves multiple macromolecules that function independently, but when brought together can effectively sense damaged DNA. This is referred to as 'matchmaking', and replication factor C (RFC) and xeroderma pigmentosum C (XPC) are examples of this.
Combinatorial, or multistep, recognition is most commonly found in transcriptional regulation and transcriptionally-coupled DNA damage repair. Combinatorial recognition is meant in a broad sense, in that repair proteins are interdependent and act in concert to ultimately recognize damage. A specific example of this is human nucleotide excision repair in which RPA, XPA, and XPC act in a cooperative manner to achieve high specificity in recognition of DNA damage structures.

Proteins that are not directly involved in DNA repair can also function in the recognition of damaged DNA by-proxy. The best example of this is transcription-coupled repair, in which RNA polymerase progressing along the template transcribed strand encounters a lesion and acts as a target for repair proteins that subsequently facilitate rectification of the damage.

Mechanisms of DNA Repair

Cells have evolved various different mechanisms with which to effectively repair DNA damage lesions. Direct repair is perhaps the simplest of these and an example of such would be photolyase, which has already been mentioned. While photolyase is present in most mammals, it no longer functions in humans. In humans nucleotide excision repair (NER) instead fulfills the function that photolyase otherwise would.

Nucleotide excision repair repairs damaged DNA which commonly consists of bulky, helix-distorting damage, such as pyrimidine dimerization caused by UV light. Damaged regions are removed in 12-24 nucleotide-long strands in a three-step process which consists of recognition of damage, excision of damaged DNA both upstream and downstream of damage by endonucleases, and resynthesis of removed DNA region. ER is a highly evolutionarily conserved repair mechanism and is used in nearly all eukaryotic and prokaryotic cells. In prokaryotes, NER is mediated by Uvr proteins. In eukaryotes, many more proteins are involved, although the general strategy is the same.

Base excision repair (BER) repairs damage to a single nitrogenous base by deploying enzymes called glycosylases. These enzymes remove a single
nitrogenous base to create an apurinic or apyrimidnic site. Enzymes called AP endonucleases nick the damaged DNA backbone at the AP site. DNA polymerase then removes the damaged region using its 5' to 3' exonuclease activity and correctly synthesizes the new strand using the complementary strand as a template.

Double-strand breaks, in which both strands in the double helix are severed, are particularly hazardous to the cell because they can lead to genome rearrangements. Two mechanisms exist to repair double-strand breaks: non-homologous end junction (NHEJ) and homologous recombination (HR).

NHEJ, DNA Ligase IV, a specialized DNA ligase that forms a complex with the cofactor XRCC4, directly joins the two ends. To guide accurate repair, NHEJ relies on short homologous sequences called microhomologies present on the single-stranded tails of the DNA ends to be joined. If these overhangs are compatible, repair is usually accurate. NHEJ can also introduce mutations during repair. Loss of damaged nucleotides at the break site can lead to deletions, and joining of nonmatching termini forms translocations. NHEJ is especially important before the cell has replicated its DNA, since there is no template available for repair by homologous recombination. There are "backup" NHEJ pathways in higher eukaryotes. Besides its role as a genome caretaker, NHEJ is required for joining hairpin-capped double-strand breaks induced during V(D)J recombination, the process that generates diversity in B-cell and T-cell receptors in the vertebrate immune system.

Homologous recombination requires the presence of an identical or nearly identical sequence to be used as a template for repair of the break. The enzymatic machinery responsible for this repair process is nearly identical to the machinery responsible for chromosomal crossover during meiosis. This pathway allows a damaged chromosome to be repaired using a sister chromatid (available in G2 after DNA replication) or a homologous chromosome as a template. DSBs caused by the replication machinery attempting to synthesize across a single-strand break or unrepaired lesion cause collapse of the replication fork and are typically repaired by recombination.
Topoisomerases introduce both single- and double-strand breaks in the course of changing the DNA's state of supercoiling, which is especially common in regions near an open replication fork. Such breaks are not considered DNA damage because they are a natural intermediate in the topoisomerase biochemical mechanism and are immediately repaired by the enzymes that created them.

In neurodegenerative disorders such as AD, Parkinson’s disease, and ALS, the major risk factor is age itself. Microarray analysis of post-mortem human brain samples revealed that genes encoding for synaptic transmission, learning, and memory are downregulated after age 45, and that this is associated with elevated levels of oxidative damage in the promoters of the downregulated genes. In addition to this, DNA DSBs and an upregulation of DNA damage response genes precede the appearance of all other AD-like neuropathological hallmarks in CK-p25 mice, and elevated levels of DNA strand breaks were observed in the AD brain itself. Together, these results raise the intriguing possibility that the accrual of DNA damage with age could underlie the pathological changes associated with neurological disease. Interestingly, SIRT1 is known to directly modulate synaptic plasticity and memory formation, and SIRT1 redistribution in response to chronic DNA damage is thought to underlie some of the transcriptional changes in the aging brain. Considering these observations therefore, the benefits conferred by pharmacological activation of SIRT1 could be significant.
CHAPTER 2: SIRT1 and HDAC1 are Functional Interaction Partners

Matthew M Dobbin
Summary

In order to assess whether or not HDAC1 and SIRT1 are in any way functionally involved in the neuronal DNA damage response in a cooperative manner, we first sought to determine whether the two interact. Importantly, HDAC1 and SIRT1 were found to interact endogenously, in neural tissue, neuronal cells, and also non-neuronal cells. Furthermore, we mapped the domain in which HDAC1 interacts with SIRT1 to the N-terminus of HDAC1 by fragmenting HDAC1 into functional domains and assessing their interaction. Using an in-vitro reconstituted protein system, the interaction between HDAC1 and SIRT1 was verified.

We then sought to dissect the functional relevance of the interaction of HDAC1 and SIRT1. As revealed by quantitative mass spectrometry, SIRT1 was shown to be capable of deacetylating six specific lysine residues on HDAC1. SIRT1 targets one specific lysine residue on HDAC1 in particular, lysine 432 (K432) in both in-vitro and in-vivo systems. We then created point mutations for each of the lysine residues on HDAC1 that SIRT1 deactylated. Each lysine was mutated in a way that either mimicked a state of acetylation (K-Q) or a state of being un-acetylated (K-R). Very importantly, we found that of the six lysines deacetylated by SIRT1, the acetylation state of K432 profoundly affected the enzymatic activity of HDAC1 thus demonstrating the importance and functional relevance of the interaction of HDAC1 and SIRT1.
Introduction

In addition to phosphorylation, post-translational acetylation is also known to modulate the activity of a number of proteins. This highly reversible modification occurs on the ε-amino group of lysine and changes the electrostatic properties of proteins by neutralizing the positive charges on the amino group (Glozak et al., 2005). Acetylation is regulated by the concerted actions of acetyltransferases and deacetylases, which add and remove acetyl groups, respectively. Many of these enzymes were originally identified as histone acetyltransferases (HATs) and histone deacetylases (HDACs) but were later found to possess activity towards non-histone proteins (Glozak et al., 2005). Human cells contain at least 18 different HDACs that have been categorized into four classes: Class I HDACs, which comprise HDACs1-3 and HDAC8, resemble the yeast Rpd3; Class II HDACs, which consist of HDACs4-7, HDAC9, and HDAC10, show similarity to the yeast Hda1; Class III NAD+-dependent HDACs (also referred to as sirtuins), constituting of SIRT1-7, show similarity to yeast Sir2; and Class IV HDACs, which are represented by a single member, HDAC11, shows some properties of both Class I and Class II HDACs (Glozak and Seto, 2007; Gregoretti et al., 2004).

SIRT1 associates with and deacetylates NBS1, and maintains it in a hypoacetylated state, which is required for ionizing radiation-induced phosphorylation and activity of NBS1 (Yuan et al., 2007). Similarly, SIRT1 deacetylates Ku70 and the WRN protein, and stimulates their enzymatic activities in DNA repair, and deacetylates p53 to suppress apoptosis and promote cell survival (Jeong et al., 2007; Li et al., 2008; Vaziri et al., 2001). SIRT1 was also recently shown to deacetylate XPA and facilitate the repair of UV-induced DNA damage (Fan and Luo, 2010).

In addition to SIRT1, human HDAC1 and HDAC2 were also shown to stimulate DSB repair through NHEJ, and to mediate the hypoacetylation of
H3K56 and H4K16 following the induction of DSBs (Miller et al., 2010). HDAC1 was also shown to physically interact with ATM (Kim et al., 1999).

In this chapter, I will describe the entirely novel observation that HDAC1 and SIRT1 physically interact with each other, and that this interaction is strengthened upon DNA damage. Furthermore, SIRT1 deacetylates HDAC1 and stimulates its enzymatic activity.
Experimental Procedures

**Binding of HDAC1-flag with SIRT1-his**
Anti-flag-conjugated agarose beads (200μl slurry; Sigma) were washed with 0.1M Glycine (pH 3.5) and resuspended in 100μl binding buffer (50mM Tris-HCl [pH 7.5 at 25°C], 5mM MgCl₂, 1mM EDTA, 100mM NaCl, 0.05% NP-40, 10% glycerol). HDAC1-flag and SIRT1-his (1μg each reaction) were combined in a reaction buffer containing 50 mM Tris-HCl [pH 8.0 at 25°C], 1mM DTT, 137mM NaCl, 2.7mM KCl, 4mM MgCl₂, 0.1mM EDTA, 1mM NAD⁺, and 10% glycerol (30μl total volume), and incubated at 37°C for 1h. Each reaction was then supplemented with 30μl bead slurry and 90μl of binding buffer, and incubated in a rotator overnight at 4°C. Following this, the beads were pelleted, washed twice with 150μl binding buffer, boiled in Laemmli SDS-PAGE loading dye, and analyzed by 8% SDS-PAGE.

**Acetylation/Deacetylation reactions and measurement of HDAC1 enzymatic activity**
In acetylation reactions (30μl), 100ng recombinant HDAC1-flag was incubated together with indicated amounts of recombinant p300-HA in a buffer containing 50mM Tris [pH 8.0 at 25°C], 1mM DTT, 1mM PMSF, 0.1mM EDTA, 50nM acetyl-CoA, and 10% glycerol were incubated at 30°C for 1h. Following this, the reaction products were then loaded onto 10% SDS-PAGE, electrophoresed, and then transferred onto nitrocellulose membranes. The membranes were stained with the indicated antibodies and analyzed by quantitative western blotting. For deacetylation reactions, acetylation reactions were first performed using 100ng HDAC1-flag and 50ng p300-HA as described above. The reactions were then supplemented with NaCl (137mM final), KCl (2.7mM final), MgCl₂ (4mM final), NAD⁺ (1mM final), and the indicated amounts of recombinant SIRT1-his, and further incubated for 1h at 37°C. Deacetylation of HDAC1 was analyzed using western blotting as described above.

For the measurement of HDAC1 enzymatic activity, acetylation and deacetylation reaction mixtures were subjected to dialysis to remove acetyl CoA.
and NAD\(^+\) (and thereby inactivate p300 and SIRT1, respectively). To further ensure SIRT1 inactivation, nicotinamide (NAM; 5mM final) was added after dialysis. HDAC1 enzymatic activity was then measured using a fluorometric assay as described in Haumaitre et al. (2008).

**Mouse Strains, Expression constructs, shRNA constructs, and virus generation**

Hdac1 F/F and Sirt1 F/F mice were the kind gifts of Dr. Eric N. Olson and Dr. Frederick W. Alt, respectively and were as described (Cheng et al., 2003; Montgomery et al., 2007). Murine HDAC1 and SIRT1 were sub-cloned into pcDNA6.2/C-EmGFP Gateway Vector (Invitrogen cat. no. V355-20) and pcDNA6.2/N-EmGFP Gateway Vector (Invitrogen cat. no. V356-20), resulting in C-terminal and N-terminal fusion proteins respectively. HDAC1 fragments were constructed according to functional protein domains as determined by bioinformatic analysis with PFAM and as described previously (Yang and Seto, 2008). The Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene cat. no. 200518) was used to generate HDAC1 mutants mimicking either a constitutively acetylated or non-acetylatable state at amino acid position 432 (K432Q and K432R, respectively). Because fusion of several different affinity tags to the N-terminus of HDAC1 was shown to interfere with its catalytic activity, all HDAC1 fusion constructs were generated as C-terminal fusions. HDAC1 and SIRT1 shRNA constructs and catalytic residue mutants were as previously described (Kim et al., 2008; Kim et al., 2007). The HA-ER-I-Ppol overexpression construct was obtained from the lab of Dr. Michael Kastan (St. Jude’s Children’s Research Hospital) (Berkovich et al., 2007, 2008), and was modified for production of lentivirus by subcloning into a lentiviral backbone containing T2A-RFP under the control of the PGK promoter. Lentiviral constructs, lenti-Cre and lenti-ΔCre, were the kind gift of Dr. Richard Huganir (Johns Hopkins University) and were as reported previously (Takamiya et al., 2008).

**Antibodies**

HDAC1 antibodies against acetylated HDAC1 (K432Ac) were generated from rabbits injected with acetylated c-terminal peptide (peptide sequence: GEGGRK(Ac)NSSNF). Antibodies used were for staining were as follows: anti-
HDAC1 1.T9 (Abcam; ab31263) 1:1000, anti-SIRT1 (Abcam; ab7343) 1:1000, anti-γH2AX (Millipore; 05-636) 1:1000, anti-flag M2 (Sigma; F1804) 1:1000. Antibodies used for western blots were as follows: anti-flag M2 (Sigma; F1804) 1:1000, anti-c-myc 9E10 (Thermo; MA1-980) 1:1000, anti-HDAC1 (Thermo; PA1-860) 1:1000, anti-Sir2α (Millipore; 07-131) 1:1000, anti-acetyl-lysine (Millipore; 05-515) 1:500, anti-HA (Millipore; ab3254) 1:1000, anti-HDAC1 (K432ac) 1:1000. Antibodies used for ChIP and IP experiments were all used at an amount of 2μg per IP reaction, and are as follows: anti-Histone H4, pan (Millipore; 04-858), anti-ATM (pS1981) (Abcam; ab36810), anti-HDAC1 1.T9 (Abcam; ab31263), anti-NBS1 (Novus; NB100-60654), anti-Sir2α (Millipore; 07-131), anti-flag M2 (Sigma; F1804).

**Cell culture and transfection/infection**

Cell lines used for experimentation were either Human Embryonic Kidney 293T cells (HEK293T) (ATCC; CRL-11268) or an immortalized mouse hippocampal cell line; HT22 (Davis and Maher, 1994). All cell lines were maintained in Dulbecco’s Modified Eagle Media (DMEM; Gibco, 10566) supplemented with L-glutamine (5mM), penicillin/streptomycin, and FBS. For live-imaging experiments, phenol red-free DMEM (Gibco; 31053) was used as a media substitute. Cell lines were maintained at standard environmental conditions (97% humidity, 5% CO₂, 37°C).

For primary neurons, dissociated cortical neurons dissected from E16-E18 Swiss-Webster mice were plated at a density of 500,000 cells/well for 24-well plates, 2 million cells/plate for 35 mm glass bottom plates, and 15 million cells/plate for 10 cm plates. The plates were coated beforehand by incubation with poly D-lysine (0.05 mg/ml) and laminin (0.005 mg/ml) for 1 hour at 37°C, followed by washing twice with dH₂O. Neurons were maintained in neurobasal media (NB) (Gibco; 21103) supplemented with L-glutamine (5mM), penicillin streptomycin, and B27 neuronal additive. Phenol red-free NB (Gibco; 12348) was used as a substitute for neuron cultures used in live-imaging experiments.
Cell lines and primary neuron cultures were transiently transfected using Lipofectamine 2000 reagent (Invitrogen; 11668) for at least 1 hour in media lacking antibiotics, after which cells were washed in warmed media and given at least 24 hours to allow for construct expression prior to usage. Virus was added directly to the media for infections. I-Ppol-ER was induced by adding 4-OHT (Sigma; H7904) to a final concentration of 1μM and incubated for 6 hours prior to fixation.

**Western blotting and immunoprecipitation**

For both primary neurons and cell lines, 1.5-2 x 10^6 cells were washed once with PBS and lysed for 10 min on ice in RIPA buffer (150 mM NaCl, 1% IGEPAL, 0.5% NaDOC, 0.1% SDS, 50 mM Tris pH 8.0, supplemented with protease inhibitors) on the plates in which they were originally grown. Cells were then collected by scraping and rotated for 30 min at 4°C. Supernatant was collected following centrifugation (13,000 rpm, 10 min, 4°C). For each sample, SDS protein loading buffer was added to 1X and boiled (95°C, 10 min) before loading onto a 10% SDS-PAGE gel. Gels were transferred to PVDF membranes (200 mA constant current) and blocked with 3% BSA in PBS-T for one hour prior to application of primary antibodies. Membranes were visualized either with electro-chemiluminescence and autoradiographic film detection, or with the LiCor Odyssey quantitative western imaging system.

For immunoprecipitations, 1mg total protein lysate was used for each condition and brought to a total volume of 500μl with RIPA buffer. The appropriate antibody was then added and the mixture was incubated on a rotator overnight at 4°C. As required, protein A/G-conjugated agarose beads (GE Health Science; 17-5280/17-0618) or flag M2 affinity gel (Sigma; A2220) were equilibrated with RIPA buffer, blocked overnight with 3% BSA, and washed prior to adding to samples. For all immunoprecipitations, a total volume of 30μl bead slurry was used per reaction mix. The reaction mixtures were incubated for 1 hour at 4°C, following which they were washed 4 times, and denatured by boiling (95°C, 10 min) in RIPA buffer containing 1X SDS sample buffer.
Computational Homology Modeling

The I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) computational protein structure prediction algorithm was used to generate a 3-dimensional predicted model for HDAC1\textsuperscript{14,15}. The mouse HDAC1 (NP_032254) amino acid sequence, and crystal structure from the ancient HDAC1 ancestor, HDLP (RCSB PDB ID: 1C3P)\textsuperscript{16}, were used as inputs for processing by the I-TASSER algorithm. Output structure coordinates were processed and formatted in PyMol, and amino acids were color-coded to correspond to the HDAC1 functional domain schematic.
Results

**HDAC1 physically interacts with the NAD\(^+\)-dependent deacetylase SIRT1**

In our efforts to understand the modes of HDAC1 regulation in the neuronal DDR, we were reminded of a previous study in which expression of the NAD\(^+\)-dependent HDAC, SIRT1, also suppressed neuronal loss in CK-p25 mice (Kim et al., 2007). Interestingly, several reports have since implicated SIRT1 in various aspects of the DDR (Jeong et al., 2007; Oberdoerffer et al., 2008; Yuan et al., 2007). From these observations, we reasoned that HDAC1 and SIRT1 might work collaboratively to maintain genomic integrity. To address this possibility, we co-overexpressed full-length, flag-tagged HDAC1 together with myc-tagged SIRT1 in HT22 cells, and found that SIRT1 co-immunoprecipitates with HDAC1 (Figure 3). Following this observation, multiple flag-tagged fragments of HDAC1 were generated (Figure 1) and the interaction was mapped to the N-terminal domain of HDAC1 (Figure 2). Interestingly, this region of HDAC1 was previously described as a protein-protein dimerization domain, mediating interactions with HDAC2, SIN3A, SIN3B, and RbAP48 (Alland et al., 2002; Hassig et al., 1997; Luo et al., 2009). Notably, flag-tagged HDAC2, which shares significant sequence similarity with HDAC1, was unable to pull down SIRT1 under these conditions (Figure 2).
FIGURE 1. Diagram illustrating HDAC1 fragment constructs for interaction mapping. FL – full-length; CTD – carboxy-terminal domain; CAT – catalytic domain; NTD – amino-terminal domain.
FIGURE 2. The indicated flag-tagged fragments of either HDAC1 or HDAC2 were expressed together with SIRT1-myc, and the flag-tagged proteins were immunoprecipitated and blotted with antibodies against myc.
Recombinant SIRT1-his was incubated with either HDAC1-flag or HDAC2-flag, following which HDAC1 and HDAC2 were precipitated with anti-flag conjugated agarose beads, and their ability to retain SIRT1 was assessed.
FIGURE 4. Recombinant SIRT1-his and HDAC1-flag were incubated either in the presence of SIRT1 inhibitors, sirtinol and nicotinamide, or in the absence of NAD+. Following this HDAC1 was precipitated and analyzed as in (Fig. 3).

FIGURE 5. Recombinant SIRT1-his and HDAC1-flag were incubated either in the presence or absence of trichostatin A (TSA) Following this, HDAC1 was precipitated and analyzed as in (Fig. 3).
FIGURE 6. Increasing amounts of p300 were incubated with a fixed amount of HDAC1 and the effect of p300 on the acetylation of HDAC1 was assessed using quantitative western blotting (* p < 0.05, one-way ANOVA).
**FIGURE 7.** Increasing amounts of SIRT1 were incubated with a fixed amount of p300 and HDAC1 and analyzed as in (Fig. 6) (* p < 0.05, one-way ANOVA).

<table>
<thead>
<tr>
<th>Condition</th>
<th>HDAC1</th>
<th>HDAC1+p300</th>
<th>HDAC1+SIRT1</th>
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<tr>
<td>SIRT1-myc</td>
<td>-</td>
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<tr>
<td>p300-HA</td>
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<td>+</td>
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<tr>
<td>HDAC1-flag</td>
<td>+</td>
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**IP: α-flag**

- α-KAc
- α-flag
- α-myc

**FIGURE 8.** HDAC1-flag, p300-HA, and SIRT1-myc were expressed as indicated in HEK293T cells. HDAC1 was immunoprecipitated, and its acetylation status was probed using an anti-acetyl lysine antibody.
FIGURE 9. HDAC1 was pre-incubated with either p300 or with SIRT1 as in (Fig. 6) and (Fig. 7), and its enzymatic activity was examined using a fluorescence based HDAC enzymatic activity assay (Experimental Procedures) (** p < 0.01, one-way ANOVA).

FIGURE 10. Schematic of a fluorescence-based reporter assay used to measure HDAC1 enzymatic activity. Deacetylation of the substrate sensitizes it to cleavage by trypsin, which results in the release of a fluorescent moiety (green). Fluorescence intensity is thus used as an indicator of deacetylase activity.
**FIGURE 11.** Sirt1 F/F neurons were infected with a lentiviral vector carrying either a functional (Cre-eGFP) or non-functional (eGFP) Cre recombinase. The infected neurons were then lysed, HDAC1 was immunoprecipitated, and its activity was measured as in (Fig. 9) (*p < 0.05, student's t-test).

**FIGURE 12.** HEK293T cells were transfected with either an empty vector or a vector carrying SIRT1. HDAC1 was then immunoprecipitated, and its activity was measured as in (Fig. 9) (*p < 0.01, student's t-test).

**FIGURE 13.** Diagram depicting the acetylated lysine residues in HDAC1 and HDAC2. Five of the six lysines are conserved between HDAC1 and HDAC2, with residue K432 in HDAC1 being substituted by R433 in HDAC2.
FIGURE 14. Recombinant SIRT1 was incubated together with p300 and HDAC1 as in (Fig. 9), and acetylation of HDAC1 was assessed using an antibody specific to acetylated K432. Quantification was performed as in (Fig. 9) (Arrowhead shows a non-specific band in the lanes containing recombinant SIRT1 that cross-reacts with the K342Ac antibody; * p < 0.05, one-way ANOVA).

FIGURE 15. Annotated MS/MS spectrum of Lys acetylated peptide "NSSNFK_kAK_R" that identified lysine acetylation sites on K438, K439 and K441 of HDAC1 upon reaction with p300. b and y ions represents collision-induced peptide fragment ions containing N- or C-terminal respectively. ** indicates fragment ions with neutral loss of amine.
FIGURE 16. Label-free quantification for each lysine acetylation site using protein-abundance normalized peptide precursor ion intensity shows that lysine acetylation abundance on p300-treated HDAC1 decreased upon the addition of SIRT1 to the reaction.
To ascertain whether HDAC1 can directly bind SIRT1, purified, recombinant HDAC1-flag, and recombinant SIRT1-his were incubated together with anti-flag conjugated agarose beads, following which the beads were washed and analyzed by SDS-PAGE. In contrast to anti-flag agarose beads alone, beads
incubated with HDAC1-flag, but not HDAC2-flag, were able to retain SIRT1, indicating a direct physical interaction (Figure 3).

To further characterize the interaction, we repeated the binding reactions either in the absence of NAD$^+$ (SIRT1 being an NAD$^+$-dependent deacetylase), or in the presence of nicotinamide (NAM), a well-established inhibitor of SIRT1. Neither omitting NAD$^+$ nor incubating the reaction in the presence of NAM had any effect on HDAC1 binding to SIRT1, indicating that the deacetylase activity of SIRT1 is dispensable for HDAC1 binding (Figure 4). We also performed the binding reactions in the presence of sirtinol, another known inhibitor of SIRT1. Interestingly, the presence of sirtinol disrupted the ability of HDAC1 to pull down SIRT1 (Figure 4). In the deacetylation reaction, NAM acts as a noncompetitive inhibitor – it attacks the ADPR-peptidyl imidate intermediate and reverses SIRT1-mediated deacetylation (Avalos et al., 2005). On the other hand, sirtinol, a competitive inhibitor, is thought to inhibit SIRT1 activity by occluding the enzyme’s active site (Westphal et al., 2007). The distinct inhibitory mechanism adopted by NAM and sirtinol could underlie their differential effects on the SIRT1-HDAC1 interaction. On the other hand, the presence of trichostatin A (TSA), a competitive inhibitor of HDAC1 that does not affect SIRT1, had no effect on SIRT1 binding (Figure 5).

Several previous reports describing an interaction between SIRT1 and components of the DDR, such as NBS1 and KU70, reported that these interactions result in the deacetylation of these proteins (Jeong et al., 2007; Yuan et al., 2007). Furthermore, HDAC1 was shown to be acetylated by the p300/CREB-binding protein (CBP) acetyltransferase and acetylation of HDAC1 inhibited its deacetylase activity (Qiu et al., 2006). We therefore examined whether SIRT1 could regulate the enzymatic activity of HDAC1 by affecting its acetylation status. First, we incubated purified, recombinant HDAC1 with increasing concentrations of recombinant p300, and recapitulated the observation that p300 acetylates HDAC1 (Figure 6). We then incubated the HDAC1-p300 reactions with increasing amounts of recombinant SIRT1, and analyzed its effect on HDAC1 acetylation by quantitative western blotting using antibodies directed against
acetyl-L-lysine. The amount of acetylated HDAC1 decreased in a dose-dependent manner in the presence of SIRT1 indicating that SIRT1 can in fact, directly deacetylate HDAC1 (Figure 7). To verify this result in cells, we expressed HDAC1-flag alone, HDAC1-flag and p300-HA, or HDAC1-flag, p300-HA, and SIRT1-myc in the hippocampal HT22 cell line, immunoprecipitated HDAC1, and assessed its acetylation status. Expression of HDAC1 together with p300 resulted in an increase in the fraction of acetylated HDAC1, whereas co-expression of SIRT1 with HDAC1 and p300 decreased the acetylation of HDAC1 (Figure 8).

To determine whether deacetylation of HDAC1 affects its enzymatic activity, we incubated either SIRT1 or p300 together with recombinant HDAC1, and examined their effect on the deacetylase activity of HDAC1 using a fluorimetric assay (Figure 10). Incubation of HDAC1 with p300 caused about a 40% reduction in HDAC1 activity, whereas incubation with SIRT1 stimulated the deacetylase activity of HDAC1 by about 30% (Figure 9). To verify this result in neurons, we immunoprecipitated HDAC1 from Sirt1 F/F neurons transduced with either eGFP or Cre-eGFP lentivirus and compared its activity. HDAC1 precipitated from Cre-eGFP expressing neurons displayed a 20% reduction in activity relative to Sirt1 F/F neurons expressing eGFP (Figure 11), indicating that SIRT1 stimulates HDAC1 activity in neurons. Conversely, overexpression of SIRT1 resulted in an increase in HDAC1 activity (Figure 12).

HDAC1 contains six acetylatable lysine residues, and notably, lysine residue 432 (K432), located near the C-terminus of HDAC1, was previously shown to be particularly important for its enzymatic activity – mutation of this residue to glutamine (K432Q) almost completely abolished HDAC1 activity (Qiu et al., 2006). Interestingly, whereas five of these six lysines are conserved between HDAC1 and the closely related HDAC2, K432 in HDAC1 is substituted by arginine in HDAC2 (Figure 13), and only HDAC1, but not HDAC2, can be acetylated by p300 (Luo et al., 2009). Based on these observations, we developed an anti-acetyl-L-lysine antibody that specifically recognizes K432 of HDAC1. We then incubated recombinant HDAC1-flag with either p300 alone or
p300 and SIRT1 as before, and examined the acetylation status at K432. Incubation with p300 increased, whereas the presence of SIRT1 decreased, the amount of HDAC1 acetylated at K432 (K432Ac; Figure 14), indicating that SIRT1 deacetylates HDAC1 at this residue. To confirm this result, we subjected the above reaction mixtures to mass spectrometry analysis and identified the acetylated residues. In the presence of p300 alone, HDAC1 was readily acetylated at lysine residues 89, 220, 412, 432, 438, 439, and 441 (Figure 15 and 16). Label-free quantification indicated that the addition of SIRT1 resulted in decreased acetylation on nearly all the lysine acetylation sites except K412 (Figure 15 and 16). Moreover, acetylation in two of these residues, K220 and K432, was not detected in the mass spectrometry assay, suggesting significant deacetylation activity of SIRT1 on the two acetyl-lysine residues (Figure 16). Furthermore, in an attempt to understand how a physical interaction of SIRT1 with the NTD of HDAC1 relates to decetylation of residues near the C-terminus, we used a previously crystallized homolog of HDAC1 from the hyperthermophillic bacterium Aquifex aeolicus (Finnin et al., 1999) as our seed template and generated a computationally predicted tertiary structure of HDAC1 (Figure 17). Interestingly, the region of HDAC1 containing K432 lies in close proximity in three-dimensional space to the NTD (Figure 17). These results suggest that the physical interaction between SIRT1 and HDAC1 results in the deacetylation of HDAC1, especially at residue K432, and that this deacetylation stimulates the enzymatic activity of HDAC1.
Discussion

Our results provide the first insights into how the activity of HDAC1 could be regulated during the DDR. We observe that HDAC1 physically interacts with SIRT1 in the presence of DNA damage, and that in the absence of SIRT1, both the retention of HDAC1 at DSBs, and its ability to promote DNA repair, are compromised (Figure 1). Furthermore, SIRT1 deacetylates HDAC1 and stimulates its enzymatic activity (Figure 5). Interestingly, we observed that HDAC1 acetylation increases in the presence of DNA damage, and the levels of this acetylation increase even further in the absence of SIRT1 (Chapter 3, Figure 3). These results suggest that SIRT1 stimulates HDAC1 activity by maintaining HDAC1 in a hypoacetylated state in response to DNA damage.

Of the four members of the Class I family of HDACs, HDAC1 and HDAC2 are highly related proteins, sharing ~85% sequence identity (Segre and Chiocca, 2011). This structural similarity extends to redundant functions in various biological processes, including differentiation of hematopoietic and neuronal precursors, cell cycle progression, and DNA repair (Miller et al., 2010; Segre and Chiocca, 2011; Yamaguchi et al., 2010). Despite these similarities, we observed that only HDAC1, but not HDAC2, physically interacts with SIRT1 in vitro and in vivo. This observation fits nicely with the recent finding that unlike HDAC1, HDAC2 cannot be acetylated by p300 even though five of the six lysines that can be acetylated in HDAC1 are conserved in HDAC2 (Luo et al., 2009). The unique lysine in HDAC1, K432, is also the most critical for the regulation of HDAC1 enzymatic activity through acetylation, and we show that SIRT1 specifically deacetylates HDAC1 at K432 both in vitro and in vivo. Moreover, an acetyl mimetic HDAC1K432Q mutant induced DNA DSBs in neurons, and unlike in control neurons, SIRT1 overexpression was unable to reverse the sensitivity of K432Q expressing neurons to DNA damaging agents (Chapter 3, Figures 35 and 36). Although the specific functions of HDAC1 deacetylation at K432Q is not completely clear, these results define acetylation/deacetylation as an important...
switch that specifically regulates HDAC1 activity during the DDR, and identify SIRT1 as an HDAC1 deacetylase.

We observe that both SIRT1 and HDAC1 are rapidly recruited to DSB sites (Chapter 3, Figures 4 and 5), where they stabilize ATM and NBS1 (Chapter 3, Figures 6 and 7), two components that lie at the apex of the DSB signaling cascade. SIRT1 is also essential for the efficient phosphorylation of γH2AX, which amplifies the DDR and promotes DNA repair. Together, these results indicate that SIRT1 and HDAC1 are necessary for the early events triggered in response to DSB formation. Findings from several other groups support our observations: SIRT1 was shown to stimulate the activities of Ku70 and NBS1 (Jeong et al., 2007; Yuan et al., 2007); human HDAC1 and HDAC2 were shown to localize to DSBs and deacetylate H3K56 and H4K16 (Miller et al., 2010); and exposure to the HDAC inhibitor, TSA, decreased the retention of NHEJ factors at DSB sites (Miller et al., 2010). In addition to this, the NuRD complex, which contains both chromatin remodeling and HDAC activities, is one of the first protein complexes known to localize to DSBs (Ball and Yokomori, 2011; Lukas et al., 2011). HDAC1 is a component of NuRD, although it is unclear whether its recruitment to DSBs occurs independently or as part of this complex. An early role for HDAC1 and SIRT1 in the DDR seems to counteract the view that chromatin relaxation allows DNA repair factors to access DSB sites more easily. However, chromatin compaction in the initial stages after DSB formation could also favor the DDR by preventing DNA ends from diffusing away and by repressing transcription in the vicinity of break sites. In support, acetylation of H4K16 was found to display a biphasic response to DNA damage, with decreased acetylation at early times, and increased acetylation later, and the early recruitment of NuRD and polycomb complexes to DSBs is thought to mediate transcriptional silencing (Lukas et al., 2011; Miller et al., 2010). Our findings also attest to the increasing complexity of protein-protein interactions at DSB sites and their functions in the DDR. For instance, in a previous report, the recruitment of SIRT1 to DNA damage sites was shown to be dependent on ATM activity (Oberdoerffer et al., 2008). Here we observe that
ATM retention at DSBs also requires SIRT1 (Figure 13). On the other hand, it is intriguing that HDAC1 is essential for the stable retention of ATM and SIRT1 at DSBs, but not essential for the formation of γH2AX foci. In fact, γH2AX intensity increases in the absence of HDAC1. One possible explanation is that these factors still transiently localize to DSBs in the absence of HDAC1, and that this localization is sufficient to phosphorylate H2AX, but insufficient to promote successful DNA repair.
CHAPTER 3: SIRT1 and HDAC1 are Apical Components of the Neuronal DNA Damage Signaling and Repair Pathway, and their Cooperativity is Required for Maintenance of Genomic Integrity

Matthew M Dobbin
Summary

Although genomic integrity mechanisms have been a subject of intense investigation, much of our knowledge stems from studies conducted in proliferating cells, and not postmitotic cells such as neurons. Previously, we reported that the levels of p25, a proteolytic cleavage fragment of the Cdk5 activating partner, p35, were induced by neuronal toxicity and elevated in brains undergoing neurodegeneration (Lee et al., 2000; Patrick et al., 1999). Thereafter, we generated a bitransgenic mouse model (Ck-p25 mice) that allowed us to express p25 in the postnatal forebrain in an inducible manner and observed that this mouse model recapitulates various aspects of AD pathology, including the accumulation of amyloid-β peptides, neurofibrillary tau pathology, astrogliosis and neuronal death in the forebrain (Cruz et al., 2006; Cruz et al., 2003). However, two recent developments turned our focus towards the DNA damage response in neurons and its role in neurodegeneration – First, further characterization of CK-p25 mice revealed that the appearance of DNA DSBs and aberrant cell cycle re-entry by neurons precede all other neuropathological symptoms in these mice (Kim et al., 2008), raising the possibility that DNA damage could be an initiating event in the neurotoxicity caused by p25 generation. Second, we observed that overexpression of either SIRT1 or HDAC1 could prevent neuronal loss in CK-p25 mice (Kim et al., 2008; Kim et al., 2007). Based on these results, we decided to directly characterize whether SIRT1 and HDAC1 function in the neuronal DDR and investigate a potential relationship between their abilities to confer neuroprotection. Observations from several groups implicate sirtuins and Class I HDACs in the DDR in proliferating cells. Both SIRT1 and its yeast ortholog, Sir2, relocate from their normal binding sites on repetitive DNA elements to sites of DNA DSBs and promote DNA repair (McAinsh et al., 1999; Oberdoerffer et al., 2008). SIRT1 associates with and deacetylates NBS1, and maintains it in a hypoacetylated state, which is required for ionizing radiation-induced phosphorylation and activity of NBS1 (Yuan et al.,
Similarly, SIRT1 deacetylates Ku70 and WRN protein, and stimulates their enzymatic activities in DNA repair, and deacetylates p53 to suppress apoptosis and promote cell survival (Jeong et al., 2007; Li et al., 2008; Vaziri et al., 2001). SIRT1 was also recently shown to deacetylate XPA and facilitate the repair of UV-induced DNA damage (Fan and Luo, 2010). In addition to SIRT1, human HDAC1 and HDAC2 were also shown to stimulate DSB repair through NHEJ, and to mediate the hypoacetylation of H3K56 and H4K16 following the induction of DSBs (Miller et al., 2010). HDAC1 was also shown to physically interact with ATM (Kim et al., 1999). However, the precise roles of these deacetylases in the DDR are not fully understood.
Introduction

Observations from several groups implicate sirtuins and Class I HDACs in the DDR in proliferating cells. Both SIRT1 and its yeast ortholog, Sir2, relocate from their normal binding sites on repetitive DNA elements to sites of DNA DSBs and promote DNA repair (McAinsh et al., 1999; Oberdoerffer et al., 2008). Like SIRT1, human HDAC1 and HDAC2 were also shown to stimulate DSB repair through NHEJ, and to mediate the hypoacetylation of H3K56 and H4K16 following the induction of DSBs (Miller et al., 2010).

However, the precise roles of Class I HDACs in the DDR are poorly understood. In contrast to proliferating cells, the mechanisms that preserve genomic integrity in postmitotic neurons remain largely obscure.

In previous studies carried out by our lab the p25/Cdk5 transgenic mouse model (CK-p25 mice), which recapitulates many of the pathological aspects of Alzheimer’s disease, was used to make the observation that elevated levels of DNA damage and aberrant cell cycle re-entry preceded other signs of neurotoxicity and neuronal death in these mice (Cruz et al., 2006; Cruz et al., 2003; Kim et al., 2008). These effects were found to be mediated through the inhibition of HDAC1 activity – overexpression of HDAC1 suppressed neuronal loss in CK-p25 mice, whereas either knockdown or pharmacological inhibition of HDAC1 alone caused a significant accrual of DSBs and increased cell cycle activity in neurons (Kim et al., 2008). In a separate report also from The Tsai Lab, SIRT1 overexpression was shown to also prevent neuronal loss in CK-p25 mice, although the mechanisms underlying this protection were unclear (Kim et al., 2007).

In this chapter I will describe my findings that place both HDAC1 and SIRT1 within the apical component of the neuronal DNA damage response. Specifically, the entirely novel discovery that both HDAC1 and SIRT1 are directly
recruited to DNA double-strand breaks in neurons where the subsequently function cooperatively to coordinate DSB signaling and ultimately DNA repair.
Experimental Procedures

Microirradiation and live-imaging
Microirradiation was generally performed as described previously (Kruhlak et al., 2009). All cells used for live imaging were cultured on 35mm glass-bottom culture dishes (MatTek), and maintained in a closed environmental system (97% humidity, 5% CO₂, 37°C). Transfected cells were given at least 24 hours to allow for construct expression. A Zeiss Axiovert200 inverted laser scanning confocal microscope equipped with a Zeiss LSM510 scanning head was used for all microirradiation experiments, and fluorescent-tagged proteins were imaged using a 63X, 1.4NA Plan-Apo oil-immersion objective at 4X digital magnification. Cells were first incubated with 100nM Hoechst 33242 (Sigma) for 30 min following which they were washed once with media lacking phenol red, and allowed to recover for 10 min prior to imaging. A 2μm² ROI was selected within each cell for microirradiation. With a 488 HFT dichroic in place, a 405 nm diode laser set to 100% transmission was used to scan the selected ROI for 25 iterations. A 509 nm band-pass filter was used for light collection, and image acquisition and frame rates were adjusted to optimally accommodate the unique dynamics of different fluorescent-tagged proteins. For each condition, data was collected from at least twenty cells. To allow for identification, cells used in immunostaining experiments were grown on gridded glass-bottom dishes (MatTek) and were fixed with 4% PFA 10 min prior to microirradiation. Details of image processing and analysis are provided in Supplementary Information.

FACS Analysis
DR-GFP U2OS cells were transfected at 70% confluency with the indicated constructs together with an eBFP construct, which was used as an indicator of transfection. After allowing a minimum of 36 h for expression, cells were collected and fixed in ice-cold ethanol for 1 h, washed with ice-cold PBS and strained. Cells were sorted at the MIT Flow Cytometry Facility on a BDS.
FACSDiva machine. For each condition, at least $10^5$ gated eBFP$^+$ cells were subsequently assessed for eGFP expression.

**Chromatin Immunoprecipitation**
Details of I-Ppol mouse genomic targets and ChIP primer design are described in Supplementary Information. For ChIP, $1.5 \times 10^6$ cells were subjected to a two-step dual cross-linking as described previously (Nowak et al., 2005; Zeng et al., 2006). After washing once with PBS, the NHS-ester cross-linker, disuccinimidyl-glutarate (ProteoChem; 2mM final) was added directly to the culture media, and incubated at room temperature with gentle rotation for 45 min. Plates were washed once with PBS, and replaced with 10ml PBS containing protease and phosphatase inhibitors. To this, 37% molecular biology-grade formaldehyde (Sigma) was added to a final concentration of 1% v/v and incubated at room temperature with gentle rotation. Formaldehyde was then quenched with 1M glycine, and cells were lysed using a buffer containing 150mM NaCl, 0.5%IGEPAL supplemented with protease and phosphatase inhibitors. Following centrifugation (12000 x g, 10 min), nuclei contained in the supernatant were collected and lysed in RIPA buffer (150mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8.0, supplemented with inhibitors) at 4°C for 30 min. Purified chromatin contained in the supernatant was collected and subsequently sonicated using a Branson Sonifier set to 50% duty, 40% amplitude for three repetitions of the following pulse sequence: 5sec pulse, 30sec wait, 5sec pulse, 30 sec wait, 5sec pulse. Sonicated chromatin was run out on a 0.5% agarose gel to ensure that chromatin was appropriately sheared to an average size of 300-600bp in length. A total of 50μl sonicated chromatin, diluted in RIPA buffer was used for each reaction and rotated overnight at 4°C along with the desired antibody. Protein-A or protein-G conjugated magnetic beads (Invitrogen) were equilibrated in inhibitor-supplemented RIPA buffer, and then incubated overnight at 4°C, while rotating, along with 3% BSA and tRNA from baker’s yeast (Sigma) as a carrier. After washing, 30μl beads were added to each immunoprecipitation reaction and incubated for 1h at 4°C with rotation.
After a series of washes, immunoprecipitated complexes were dissociated from the beads in TE buffer containing 200mM NaCl and 1µg proteinase K at 65°C for 6 hours, followed by phenol-chloroform extraction. Purified DNA was analyzed by quantitative-PCR using a BioRad CFX-96 quantitative thermocycler, and SsoFast EvaGreen Low-ROX qPCR SuperMix (BioRad; 172-5212). Data was processed and analyzed using the ΔΔC\text{\textsubscript{T}} method (Applied Biosystems; http://docs.appliedbiosystems.com/pebiodocs/04371095.pdf) to determine the differential occupancy for each immunoprecipitated protein.

**Single cell electrophoresis (comet) assays**
Cultured primary neurons of indicated genotypes were treated with 5µM etoposide for 1h and either allowed to recover for 24h or dissociated immediately using 0.025% Trypsin-EDTA. The dissociated neurons were embedded in a thin layer of low-melting agarose (0.5%). Lysis, alkaline treatment, and single-cell gel electrophoresis under alkaline conditions were performed as described in (Singh et al., 1988).

**In-vivo drug administration**
Animals were administered with oral doses of 30 mg/kg SRT3657 for 4 weeks. Oral gavage was performed using 1.5-inch, curved, 20-gauge, stainless steel feeding needles with a 2.25-mm ball (Braintree Scientific, Braintree, MA). Twice-daily gavage treatments were performed between the hours of 0800 to 1000 and 1600 to 1900.
**Results**

To determine whether SIRT1 and HDAC1 function in the DSB response in neurons, we treated cultures of dissociated cortical neurons from E16 mouse embryos with the DSB-inducing drug, etoposide, and analyzed the sub-nuclear localization of SIRT1 and HDAC1 relative to γH2AX, a well-established marker of DNA DSBs. In the absence of DNA damage, both SIRT1 and HDAC1 exhibited a uniform, punctate distribution exclusive of chromocenters (sub-nuclear compartments consisting of densely packed chromatin) and nucleoli (Figure 1). In the presence of etoposide, a significant fraction of SIRT1 and HDAC1 puncta co-localized with γH2AX foci (Figure 1), indicating the presence of SIRT1 and HDAC1 at sites of DNA DSBs in neurons.

To test whether SIRT1 and HDAC1 are actually recruited to DSBs, we utilized a confocal microscope equipped with a 405 nm diode laser to irradiate a thin strip within the nuclei of individual Hoechst-stained primary neurons and visualized the resulting lesions by immunostaining for γH2AX (Figure 3, S1B). Following laser microirradiation, both SIRT1 and HDAC1 exhibited strong enrichment within lesioned regions, as marked by γH2AX (Figure 2). To verify these findings using a different approach, we employed a genetically encoded system that allowed for the inducible generation of DSBs at defined loci within the mouse genome (Figure 6). Briefly, this system consists of the rare-cutting homing endonuclease I-Ppol, fused to a mutant estrogen receptor that can only be activated by the cell permeable ligand 4-hydroxytamoxifen (4-OHT). Once activated, I-Ppol is freed to bind to, and cleave consensus target motifs. Following the induction of I-Ppol, we assessed the presence of SIRT1 and HDAC1 at a unique I-Ppol cleavage site by chromatin immunoprecipitation (ChIP). As reported previously (Berkovich et al., 2007, 2008), we detected an increase in phosphorylated (and activated) ATM levels at the I-Ppol cleavage site upon addition of 4-OHT compared to a genomic region lacking an I-Ppol cleavage site (Figure 5). Importantly, SIRT1 and HDAC1
recruitment was also enriched at the I-PpoI site following DSB generation, with ATM, SIRT1, and HDAC1 displaying the strongest accumulation immediately 3' to the cleavage site (Figure 5). In contrast, no such enrichment was detected for the core histone, H4. Together, these results suggest that SIRT1 and HDAC1 are recruited to sites of DNA DSBs in postmitotic neurons.

To further explore the dynamics of SIRT1 and HDAC1 recruitment to sites of DNA DSBs, we micro-irradiated Hoechst-stained primary neurons expressing either EmGFP-SIRT1 or HDAC1-EmGFP, and monitored the dynamics of their localization to sites of laser-induced DNA damage as a function of time (Figure 3). An increase in EmGFP-SIRT1 in the areas of laser-induced DNA damage was detected almost immediately after damage induction ($T_{1/2} = 3.21 \pm 0.48$ s; x 7 and 8), whereas HDAC1-EmGFP accumulation became apparent by ~ 60 s ($T_{1/2} = 57.7 \pm 5.8$ s) and persisted until at least 10 min after DNA damage induction (Figures 7 and 8). These results indicate that both SIRT1 and HDAC1 are recruited to DSBs rapidly in neurons, with SIRT1 displaying slightly faster kinetics than HDAC1.
FIGURE 1. Primary cortical neurons were treated with 2µM etoposide for 1h, and then fixed and stained with antibodies against either SIRT1 or HDAC1, and γH2AX as indicated. Right-most panel illustrates colocalization analysis (Intensity Correlation Analysis, ICA). Pixels from input channel co-varying positively with corresponding signal from γH2AX channel are indicated by yellow colors, while negatively co-varying pixels are indicated in blue. Scale bar = 3µm.
FIGURE 2. Primary cortical neurons were subjected to sub-nuclear, laser-generated DNA lesioning using a confocal microscope equipped with a 405 nm laser and stained with antibodies against either SIRT1 or HDAC1, and γH2AX. Scale bar = 3μm. See also (Fig. 3) and (Fig. 4).

FIGURE 3. Schematic of laser microirradiation. A Zeiss LSM710 inverted laser scanning confocal microscope equipped with a 405nm diode laser was used to irradiate a thin sub-nuclear strip of Hoechst-stained primary neurons. Localization of proteins to sites of laser-induced DNA DSBs can be monitored as increased fluorescence intensity within lesioned regions as visualized either by immunocytochemistry of fixed cells (for instance, γH2AX) or through live imaging of cells carrying fluorescently tagged repair proteins.
FIGURE 4. Generation of highly localized sub-nuclear DNA damage using laser microirradiation. Serial attenuation of transmitted 405 nm wavelength light emitted from a continuous-wave diode laser yields a dose-dependency in γH2AX signal intensity within lesion ROIs. ROI area (2μM²), laser power (100%), and scan iterations were held constant for Hoechst33242 pre-sensitized neuronal nuclei, while percent transmission was varied as indicated. Neurons were then fixed and stained with γH2AX to indicate DNA damage. γH2AX quantification was limited to lesion ROI exclusively. Scale bar = 7μM.
FIGURE 5. An engineered construct containing the rare-cutting homing endonuclease, I-Ppol, was used to generate DNA DSBs at defined genetic loci in mouse primary cortical neurons. Recruitment of the indicated proteins at a unique cleavage site located between exons 2 and 3 in the Dnahc7b gene was then assessed by chromatin immunoprecipitation (ChIP). Primers were designed at regular 1kb intervals, spanning 10kb both 3' and 5' to the I-Ppol consensus site (red dotted line). See also (Fig. 6).
FIGURE 6. Distribution of I-Ppol consensus sites across the mouse genome. Ideogram visualization of all mouse chromosomes, with I-Ppol consensus sites indicated. Genomic regions containing the Dnahc7b and Rna28s1 genes that were targeted for quantitative ChIP analysis are enlarged, with cleavage sites denoted with red arrows. Genes encoding ribosomal subunits, including Rna28s1, comprise the physical wall of the nucleolus and can be identified morphologically as DAPI-sparse stained nuclear regions (image inset). Ring-like accumulation of I-Ppol-ER-HA with surrounding γH2AX signal accumulation along the wall of the nucleolus (inset magnification) following 4-OHT induction indicates successful activation. Scale bar = 5μM.
FIGURE 7. Time-lapse images of primary cortical neurons that were transfected with a vector carrying nuclear-eGFP, HDAC1-EmGFP, or SIRT1-EmGFP, and subjected to sub-nuclear, laser-generated DNA lesioning using a confocal microscope equipped with a 405 nm laser; See (Fig. 3). Red boxes indicate damage ROI. Scale bar = 5 \mu m.
FIGURE 8. Quantification of relative fluorescence intensity ($I_{REL}$) as a function of time at lesioned ROIs (top) for neurons expressing nuclear-eGFP (grey; $n=13$, **$r=0.37$ Pearson corr.), HDAC1-EmGFP (blue; $n=15$, ***$r=0.98$ Pearson corr.), or SIRT1-EmGFP (green; $n=11$, ***$r=0.77$ Pearson corr.). Modeling and regression analysis of time-lapse data (bottom). Empty circles (green-SIRT1; blue-HDAC1) indicate data from a single trial plotted against the fitted curve (solid lines).
FIGURE 9. Relative fluorescence intensity (I_{rel}) as a function of time at lesioned ROIs for neurons expressing either EmGFP-SIRT1 (Left) or HDAC1-EmGFP (Right) together with the indicated siRNAs (* denotes p < 0.05; one-way ANOVA).
FIGURE 10. Table indicating the time taken by EmGFP-SIRT1 (left) and HDAC1-EmGFP (right) to attain half-maximal fluorescence intensity ($\tau_{1/2}$) in the lesioned region following the knockdown of DSB response components in (Fig. 9). This value was calculated following modeling and regression analysis of time-lapse data in (Fig. 9), as depicted in (Fig. 8).

<table>
<thead>
<tr>
<th>siRNA</th>
<th>$\tau_{1/2}$ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scrambled</td>
<td>2.82 ± 0.79</td>
</tr>
<tr>
<td>MRE11</td>
<td>1.93 ± 1.12</td>
</tr>
<tr>
<td>RAD50</td>
<td>1.80 ± 0.52</td>
</tr>
<tr>
<td>NBS1</td>
<td>3.75 ± 0.44</td>
</tr>
<tr>
<td>ATM</td>
<td>16.60 ± 1.41</td>
</tr>
<tr>
<td>KU70</td>
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</tr>
<tr>
<td>KU80</td>
<td>2.99 ± 0.71</td>
</tr>
<tr>
<td>HDAC1</td>
<td>2.87 ± 0.42</td>
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<tr>
<td>CHD4</td>
<td>2.16 ± 0.40</td>
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<table>
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<th>siRNA</th>
<th>$\tau_{1/2}$ (sec)</th>
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<tr>
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<tr>
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<tr>
<td>ATM</td>
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<tr>
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<tr>
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<tr>
<td>SIRT1</td>
<td>85.51 ± 15.04</td>
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<tr>
<td>CHD4</td>
<td>133.80 ± 25.70</td>
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</tbody>
</table>

FIGURE 11. Representative images of EmGFP-SIRT1 (top) and HDAC1-EmGFP (bottom) expressing neurons that were transfected with the indicated siRNAs to show that the various siRNAs did not affect expression of the two proteins.
FIGURE 12. Sirt1 F/F neurons were infected with lentiviral vectors carrying either a functional Cre recombinase (Cre-eGFP) or a non-functional Cre (eGFP) were treated with 5μM etoposide for 1h, and were either allowed to recover for 16h in the absence of etoposide or lysed immediately. The extent of DNA damage was then assessed using the comet assay. Graph indicates a comparison of “comet tail moments” (***p< 0.001, one-way ANOVA).

FIGURE 13. Hdac1 F/F neurons were infected and treated with etoposide as in (Fig. 12) and DNA damage was assessed using the comet assay (***p< 0.001, one-way ANOVA).
FIGURE 14. Schematic for generating Sirt1 KO neurons. Dissociated primary cortical neurons were cultured from E16 Sirt1 F/F embryos. DIV3 cultures were infected with a lentiviral vector carrying either a functional Cre recombinase (Cre-eGFP) or a non-functional Cre (eGFP). Neurons were usually used 7 days after infection with Cre-eGFP. A similar strategy was used to generate Hdac1 KO neurons.

FIGURE 15. Cultured primary neurons were transfected with a pre-digested NHEJ reporter construct (see also Fig. 16 + Fig. 17) together with scrambled shRNA, SIRT1 shRNA or HDAC1 shRNA and the number of GFP+ cells were assessed as a measure of successful DNA repair through NHEJ (* p < 0.05, one-way ANOVA).
FIGURE 16. Scheme of NHEJ reporter assay. The figure has been adapted from a previous report (Seluanov et. al., 2004) and depicts the construct used to measure efficiency of DNA repair using NHEJ. In this construct, a functional eGFP gene is interrupted by an intron. The presence of an adenoviral exon sequence within the intron prevents it from normally being spliced out. Generation of a DSB using HindIII and its subsequent repair using NHEJ disrupts the adenoviral exon sequence. Consequently, the intron is spliced out, allowing for expression of GFP. In this way, GFP+ cells can be used to score the efficiency of NHEJ.

FIGURE 17. Experimental design and quantification and scoring strategy for the NHEJ assay.
FIGURE 18. The rare-cutting homing endonuclease, I-Ppol, was used to engineer DSBs at defined regions in Sirt1 F/F neurons infected as in (Fig. 12), and the recruitment of ATM (pS1981) and NBS1 to cleavage sites within the 28SrDNA locus was assessed by ChIP as in Fig. 5 (see also Fig. 6).

FIGURE 19. The recruitment of ATM (pS1981) and NBS1 to I-Ppol-generated DSBs in Hdac1 F/F neurons infected as in (Fig. 12) were analyzed by ChIP as in Fig. 5 (see also Fig. 6).

FIGURE 20. Sirt1 F/F neurons infected as in (Fig. 12) were treated with either vehicle or 5μM etoposide for 1h, following which the cells were lysed and levels of phosphorylated ATM were compared by quantitative western blotting (*p < 0.05, student’s t-test).
FIGURE 21. The effect of the loss of HDAC1 on the levels of phosphorylated ATM and γH2AX in etoposide-treated neurons were measured using western blotting as in (Fig. 20).

FIGURE 22. Sirt1 F/F neurons infected as in (Fig. 12) were treated with either vehicle or 2μM etoposide for 1h, following which the cells were fixed and stained with antibodies to γH2AX. Per-Cell γH2AX intensity was then quantified using Cell-Profiler automated image processing software (**p < 0.01, student’s t-test).
FIGURE 23.  *Hdac1 F/F* neurons infected as in (Fig. 12) were treated with either vehicle or 2μM etoposide for 1h, following which the cells were fixed and stained with antibodies to γH2AX. Per-Cell γH2AX intensity was then quantified as in (Fig. 22) (**p < 0.01, student's t-test).

FIGURE 24.  Primary cortical neurons (DIV14) were incubated overnight with the SIRT1 inhibitor, sirtinol (20μM final), then treated with 2μM etoposide for 1h and analyzed as in (Fig. 22). Scale bar = 10μm.
FIGURE 25. Primary cortical neurons (DIV14) were incubated overnight with an HDAC1/2 specific inhibitor, BRD6929 (5μM final). Neurons were then treated with 2μM etoposide for 1 h and stained as in (Fig. 24). Scale bar = 10μm.

FIGURE 26. Mouse primary neurons transfected with the indicated HDAC1 constructs were treated with 2μM etoposide for 1 h, and γH2AX intensity was quantified using per-cell image analysis. Analysis was limited to transfected cells exclusively. Scale bar = 5μm.
FIGURE 27. U2OS cells containing a reporter sequence that can be cleaved by the I-Scel homing endonuclease was used to assess the recruitment of SIRT1 and HDAC1 to DSB sites (see Fig. 28) (** p < 0.01, one-way ANOVA).
FIGURE 28. A U2OS cell line containing an integrated reporter system, hrpt-DR-GFP, was used to assess the role of SIRT1 and HDAC1 in HR-mediated DSB repair. This system consists of two inactive eGFP genes, one of which contains an 18bp insertion that can be cleaved by the rare-cutting restriction endonuclease, I-Scel, while the other serves as a template for repair through HR. Successful repair reconstitutes a functional eGFP, and the frequency of this event can be measured by flow cytometry (left). Cells were transfected with vectors carrying I-Scel together with the indicated constructs, and the percentage of GFP+ cells was assessed under the indicated conditions using FACS (right). (* p < 0.05, one-way ANOVA).

FIGURE 29. HT22 cells were treated with camptothecin (CPT; 1μM) for the indicated times. Cells were lysed, precipitated with antibodies against HDAC1, and blotted with antibodies against SIRT1 (* p < 0.05, one-way ANOVA).
**FIGURE 30.** Quantification of relative fluorescence intensity (I_rel) as a function of time at lesioned ROIs for neurons expressing either EmGFP-SIRT1 together with HDAC1 siRNA (left) or HDAC1-EmGFP together with SIRT1 siRNA (right) (**p < 0.05, student's t-test).
**FIGURE 32.** Primary neurons cultured from SIRT1 F/F embryos were infected as in (Fig. 12). The neurons were then treated with 5pM etoposide for 1 h and the acetylation of HDAC1 at K432 was assessed using western blots.

**FIGURE 33.** Cultured primary neurons (DIV7) were treated with 5pM etoposide for 30 min. Cells were then lysed either immediately or following recovery after etoposide washout for the indicated times. The lysates were electrophoresed and the indicated acetylation marks were probed using western blots.
FIGURE 34. 293T cells were incubated overnight with sirtinol (20 μM), treated with 5 μM etoposide for 1 h, and the acetylation of HDAC1 at K432 was assessed as in (Fig. 32).
FIGURE 35. Primary neurons were transfected with the indicated vectors and treated with etoposide (2µM) for 1 h, after which the cells were fixed and stained with antibodies against γH2AX. Scale bar = 10µm. Quantification shown below (* p < 0.05, *** p < 0.001, one-way ANOVA).
Cultured primary neurons expressing flag-SIRT1 together with either HDAC1-EmGFP or HDAC1K432Q-EmGFP were treated with etoposide (2μM) for 1 h, following which the cells were fixed and stained as in (Fig. 35). Scale bar = 15μm. Quantification shown below (* p < 0.05, one-way ANOVA).
FIGURE 37. HT22 cells were transfected with the indicated constructs. Cells were then lysed and eGFP-tagged proteins were immunoprecipitated and blotted with antibodies against myc.

FIGURE 38. The activity of recombinant HDAC1-flag (100ng) incubated either in the presence or absence of the SIRT1 activator, SRT3657 (1.1μM final), was measured using a fluorescence-based HDAC enzymatic activity assay as in (Chapter 2, Fig. 9).

FIGURE 39. Cultured primary neurons expressing either SIRT1 together with HDAC1 shRNA or HDAC1 together with SIRT1 shRNA were transfected with the pre-digested NHEJ reporter construct and the number of GFP+ cells were assessed as a measure of successful DNA repair using NHEJ (see Fig. 17) (*p < 0.05, one-way ANOVA).
FIGURE 40. Indicated amounts of the SIRT1 activator, SRT3657, was incubated together with 100ng HDAC1 and 80ng SIRT1, and HDAC1 acetylation at K432 was assessed as in (Fig. 33). The use of fluorescent antibodies allows the presentation of an overlay. Arrowhead indicates a non-specific band in the recombinant SIRT1 that cross-reacts with the K432Ac antibody.

FIGURE 41. HEK293T cells were treated with the indicated concentrations of SRT3657 for 12 h. The cells were then lysed and the HDAC1 acetylation at K432 was assessed as in (Fig. 33).
FIGURE 42. Neurons incubated with 10μM SRT3657 for 12 h were treated with 5μM etoposide for 1 h, and fixed and stained with antibodies against γH2AX. Graph indicates quantification of γH2AX intensity. Scale bar = 10μm. Quantification shown below (***, p < 0.001, one-way ANOVA).
FIGURE 43. Cultured primary neurons expressing the indicated proteins were incubated with either vehicle or SRT3657. The neurons were then treated with etoposide (2μM) for 1 h, following which the cells were fixed and stained as in (Fig. 42) Quantification shown below (* p < 0.05, one-way ANOVA).
**FIGURE 44.** 6-weeks induced CKIIα-p25 mice were administered 30mg/kg SRT3657 as described in Experimental Procedures. Brains were then sectioned and stained with antibodies against γH2AX and NeuN. Graphs indicate the number of γH2AX-positive (left) and NeuN-positive (right) cells in the CA1 region of the hippocampus. Scale bar = 100μm. (* p < 0.05, *** p < 0.001, student's t-test).

**FIGURE 45.** 6-weeks induced CKIIα-p25 mice were administered either vehicle or 30mg/kg SRT3657 as in (Fig. 44). Brain lysates were then prepared and then the acetylation of HDAC1 at K432 was assessed by quantitative western blot.

The generation of DNA DSBs induces a rapid mobilization of DNA damage sensors and transducers to break sites, which further coordinate and amplify the DDR. To understand how SIRT1 and HDAC1 get recruited to DSBs,
we employed siRNAs against various known sensors of DNA DSBs, including MRE11, RAD50, NBS1, KU70, KU80, and ATM, and assessed the effect of the knockdown of each of these components on the recruitment of SIRT1 and HDAC1 to sites of laser-induced DNA damage in live postmitotic neurons. Whereas knockdown of MRE11 complex components (MRE11/RAD50/NBS1) and KU70/KU80 had no effect on the dynamics of SIRT1 recruitment to DSB sites, the knockdown of ATM caused a marked decrease in both the maximal intensity, as well as the kinetics, of SIRT1 accrual at sites of laser-induced DNA DSBs (Figures 9 and 10), indicating that SIRT1 localization to DSBs is largely dependent on ATM signaling. On the other hand, ATM knockdown did not adversely affect HDAC1 recruitment to DSBs (Figure 9). In fact, HDAC1 seemed to localize to DSBs with slightly faster kinetics after ATM knockdown compared to controls (Figure 10). However, knockdown of NBS1 and KU70/KU80 significantly reduced the intensity of HDAC1 accumulating at DSBs (Figure 9). In addition, knockdown of KU70/KU80 also affected the kinetics of HDAC1 localization to DSBs (Figure 10). HDAC1 is also a component of the NuRD (nucleosome remodeling and deacetylase) complex that localizes to DSBs, and is thought to facilitate DNA repair by silencing transcription in the vicinity of damaged DNA. To determine whether HDAC1 is recruited to DSBs as part of the NuRD complex, we used siRNAs against CHD4, an integral NuRD component known to be targeted by ATM upon DSB induction, and tested its effect on HDAC1 recruitment. Loss of CHD4 severely disrupted HDAC1 localization to DSBs (Figures 9 and 10), indicating that HDAC1 is likely recruited to DSBs as part of the NuRD complex. CHD4 knockdown also had a small effect on the maximal intensity of SIRT1 accumulation (Figure 9). Thus, SIRT1 and HDAC1 are recruited to DSBs in neurons by distinct mechanisms, with SIRT1 localizing in an ATM-dependent, and HDAC1 localizing in a NuRD, NBS1, and KU-dependent manner. Knockdown of individual DSB sensors had no effect on the expression of either EmGFP-SIRT1 or HDAC1-EmGFP (Supplementary Fig. 1D).

To understand whether SIRT1 is important for DSB repair in neurons, we transduced neurons cultured from Sirt1 floxed (F/F) mouse embryos with either a
lentiviral vector carrying the Cre recombinase (Cre-eGFP) or a control virus carrying a non-functional Cre (eGFP) (Figure 14). Neurons infected with Cre-eGFP for 72 h (Sirt1 KO neurons) showed no detectable SIRT1, confirming successful genetic deletion (data not shown). The Cre-eGFP and eGFP expressing neurons were then treated with 5μM etoposide for 1 h, following which the etoposide was washed out and neurons were allowed to recover for 16 h, and DNA damage was assessed using the single cell electrophoresis assay (comet assay) (Singh et al., 1988). Even in the absence of etoposide treatment, a significant fraction of Sirt1 KO neurons displayed comet tails and upon etoposide treatment, their “tail moments” were longer than those generated by control neurons treated similarly (Figure 12). These results suggest that neurons lacking SIRT1 somehow become more susceptible to DNA strand breaks (the assay measures both single strand breaks and DSBs). After recovery for 16 h, whereas “tail moments” in control neurons were significantly reduced, Sirt1 KO neurons continued to display long comet tails, indicating a defect in DNA repair (Figure 12). We similarly performed the comet assay in Hdac1 F/F neurons infected with either Cre-eGFP or eGFP and as with SIRT1, we observed an increase in “comet tail moments” in Hdac1 KO neurons even in the absence of etoposide and an impaired recovery after DSB induction (Figure 13). These results suggest that both SIRT1 and HDAC1 are essential for DSB repair in neurons.

DNA DSBs can be repaired using one of two pathways, homologous recombination (HR) or nonhomologous end joining (NHEJ). Of these, HR is selectively utilized during S and G2 phases of the cell cycle when the sister chromatid is readily available for homology-directed repair. Because neurons are postmitotic cells, NHEJ is likely the predominant pathway of DSB repair in neurons. To test whether SIRT1 and HDAC1 are essential for NHEJ in neurons, we utilized a previously described reporter construct in which the GFP gene is interrupted by an intron (Seluanov et al., 2004) (Figure 16). DSBs can be generated through enzymatic digestion of a corresponding restriction site present only within the intron, followed by transfection of the predigested construct into cultured primary neurons. Successful repair of this DSB using NHEJ allows the
intron to be spliced out and permits the expression of GFP. Thus, the fraction of GFP+ cells can be used to indicate the efficiency of NHEJ. We transfected the predigested NHEJ reporter construct together with control shRNA, SIRT1 shRNA or HDAC1 shRNA and compared the fraction of GFP+ cells (Figure 17). Relative to neurons transfected with a control shRNA, neurons transfected with either SIRT1 shRNA or HDAC1 shRNA displayed a significant reduction in the number of GFP+ cells, suggesting that SIRT1 and HDAC1 are essential for DSB repair using NHEJ (Figure 15).

We next sought to characterize the functions of SIRT1 and HDAC1 in DSB signaling. Because both proteins localize rapidly to DSB sites, we asked whether their loss affects the interaction of early DSB signaling components with DSB sites. Again utilizing the I-Ppol system, we assessed the recruitment of NBS1 and phosphorylated ATM to DSBs in Sirt1 KO and Hdac1 KO neurons, this time targeting I-Ppol cleavage sites within the 28S ribosomal subunit, Rna28s1, for ChIP analysis. Genes encoding ribosomal subunits are arrayed across the mouse genome in over 200 copies, and comprise the physical wall of the nucleolus. Nucleolar compartments are easily distinguished as sub-nuclear regions devoid of chromatin when visualized by DNA binding dyes. Upon activation with 4-OHT, I-Ppol displayed a largely perinucleolar distribution pattern, and was surrounded by an outer ring of γH2AX, indicating successful cleavage of the rDNA repeat sequences (Figure 6). Concomitantly, the occupancy of both phosphorylated ATM and NBS1 at the 28S rDNA locus was considerably enriched in Sirt1 F/F and Hdac1 F/F neurons infected with eGFP (Figures 18 and 19). However, no such enrichment was detected at the same sites in Sirt1 KO and Hdac1 KO neurons (Figures 18 and 19, respectively), suggesting that both SIRT1 and HDAC1 are essential for the stable retention of NBS1 and ATM at DSB sites.

An important event in DSB signaling involves the auto-phosphorylation and activation of ATM, which in turn, phosphorylates its numerous targets downstream and coordinates various arms of the DDR (Jackson, 2002). Based on our observation that SIRT1 and HDAC1 stabilize ATM at DSB sites, we
assessed the levels of phosphorylated ATM in Sirt1 F/F and Hdac1 F/F neurons transduced with either eGFP or Cre-eGFP lentiviral vectors that were treated with 5μM etoposide for 1 h. Etoposide treatment triggered a robust increase in phosphorylated ATM in eGFP-infected Sirt1 F/F and Hdac1 F/F neurons, as well as in Hdac1 KO neurons (Figures 20 and 21). Interestingly, however, ATM phosphorylation was sharply reduced in etoposide-treated Sirt1 KO neurons (Figure 20). These results suggest that in addition to stabilizing ATM at DSB sites, SIRT1 is also essential for ATM auto-phosphorylation, and thereby ATM activity. Furthermore, phosphorylation of H2AX (γH2AX), an important target of ATM in the DSB response, was also reduced in etoposide-treated Sirt1 KO neurons compared to controls (Figure 22), and similar results were observed in neurons treated with the SIRT1 inhibitor, sirtinol (Figure 24). In contrast, γH2AX levels were elevated in etoposide-treated Hdac1 KO neurons compared to controls (Figure 23). Similarly, cultured primary neurons treated with etoposide either in the presence of BRD6929, an HDAC1/2/3-selective inhibitor, or when expressing a catalytically inactive Hdac1 allele, Hdac1H141Y, also displayed increased γH2AX levels compared to controls (Figures 25 and 26). Together, these results suggest that both SIRT1 and HDAC1 are essential for NHEJ-mediated DNA repair in postmitotic neurons. In addition, SIRT1 and HDAC1 also engage in synergistic interactions with the apical components of DSB signaling, including ATM and the MRE11 complex at DSB sites, with SIRT1 also stimulating ATM activity in the DSB response. Although these results implicate SIRT1 and HDAC1 in DSB signaling and DNA repair in neurons, we also verified the association of SIRT1 and HDAC1 with sites of programmed DSBs in U2OS cells (Figure 27). Additionally, this U2OS cell line contains a stably integrated reporter system, hrpt-DRGFP, for assessing DNA repair through HR (Figure 28). We utilized this system and observed that SIRT1 and HDAC1 are also essential for HR-mediated DNA repair (Figure 28). Thus, our results regarding the interactions between SIRT1, ATM, HDAC1 and NBS1, could also apply to DSB repair in proliferating cells.
The existence of an enzyme-substrate interaction between SIRT1 and HDAC1 prompted us to investigate how this interaction impacts DSB signaling and DNA repair in neurons. We began by testing how the physical interaction between SIRT1 and HDAC1 is affected by DNA damage. Cultures of HT22 cells were treated with either vehicle or camptothecin (CPT), followed by HDAC1 immunoprecipitation to assess its ability to pull down SIRT1. A modest interaction between endogenous SIRT1 and HDAC1 was detected in vehicle-treated cells (Figure 29). However, treatment with 1 μM CPT for 1 h significantly increased the binding of SIRT1 to HDAC1, and the strength of this interaction increased even further after 3 h of CPT treatment (Figure 29), indicating that the physical interaction between HDAC1 and SIRT1 is stimulated upon the induction of DNA damage.

To understand whether an increased interaction between SIRT1 and HDAC1 upon DNA damage reflects their association at DSBs, we employed siRNAs against SIRT1 and tested the effect of SIRT1 knockdown on the recruitment of HDAC1 to sites of laser-induced DNA DSBs in neurons. In the absence of SIRT1, the accumulation of HDAC1 at DSB sites was severely disrupted (Figures 30 and 10). Similarly, knockdown of HDAC1 also perturbed the recruitment of SIRT1 to DSBs. Thus, in addition to the distinct proteins that govern the localization of SIRT1 and HDAC1 to DSBs (Figure 9), the two proteins also stabilize each other at sites of DNA DSBs. To further explore this matter, we again utilized the I-Ppol system to generate DSBs in Sirtl KO primary neuron cultures and targeted the 28S rDNA locus for analysis of HDAC1 binding by ChIP. Similarly, we assessed SIRT1 binding to DSBs induced in the rDNA locus in Hdac1 KO neurons. As described before for another site (Figure 5), SIRT1 and HDAC1 levels were also enriched at the rDNA locus following I-Ppol induction in Sirt1 F/F and Hdac1 F/F neurons expressing the eGFP virus (Figure 31). In contrast, both SIRT1 and HDAC1 showed diminished binding at DSBs in the absence of each other (Figure 31). Together, these observations suggest that SIRT1 and HDAC1 reinforce the stable binding of each other at sites of DNA.
DSBs, which is also consistent with the stimulation of SIRT1 and HDAC1 binding upon DSB induction (Figure 29).

Because SIRT1 can deacetylate and stimulate HDAC1 activity, we assessed the status of HDAC1 acetylation in Sirt1KO neurons that were treated with etoposide. First, we compared HDAC1 acetylation at K432 between control and Sirt1 KO neurons in the absence of etoposide and detected no significant differences (Figure 32). Upon treatment with etoposide, control Sirt1 F/F neurons expressing eGFP showed an increase in HDAC1K432 acetylation. However, this increase was more pronounced in etoposide-treated Sirt1 KO neurons (Figure 32). Similarly, HEK293T cells treated with etoposide in the presence of the SIRT1 inhibitor, sirtinol, also showed a robust increase in HDAC1 acetylation at K432 compared to controls (Figure 34). These results suggest that the acetylation of HDAC1 is modulated in response to DSB formation and that SIRT1 maintains HDAC1 in a deacetylated state that is essential for HDAC1 activity. However, given that HDAC1 is essential for DSB repair, we found it peculiar that the acetylation of HDAC1 increases upon DSB induction. To further clarify this issue, we briefly treated cultured primary neurons with etoposide (30 min), followed by washout and recovery in etoposide-free media. Lysates were then prepared at hourly intervals, and HDAC1 acetylation at K432 was monitored as a function of time post-etoposide treatment. Compared to untreated controls, neurons treated with etoposide for 30 min showed a reduction in HDAC1 acetylation. This trend continued until about 1 h after etoposide washout, following which time, HDAC1 acetylation levels began to rise again, surpassing the acetylation levels in untreated controls by about 4 h after etoposide washout (Figure 33). This pattern of HDAC1 acetylation is also consistent with changes in the acetylation of H4K16, a previously identified HDAC1 target in the DSB response (Figure 33). Together, these results suggest that HDAC1 activity is dynamically modulated through acetylation and that SIRT1 mediates HDAC1 deacetylation in the DSB response.

To further understand the significance of HDAC1 acetylation in the DSB response, we overexpressed GFP-tagged variants of HDAC1 carrying either a
K432Q (acetylation mimetic) or a K432R (acetylation resistant) mutation in cultured primary neurons and characterized their response to etoposide treatment. As expected, etoposide exposure triggered the formation of γH2AX foci in control neurons expressing GFP alone (Figure 35). Overexpression of HDAC1-EmGFP during etoposide treatment caused a reduction in γH2AX foci, confirming previous observations (Kim et al., 2008). In contrast, neurons expressing the K432Q mutant displayed γH2AX foci even in the absence of etoposide, and a more robust increase in γH2AX intensity upon etoposide treatment (Figure 35). On the other hand, neurons expressing the HDAC1 (K432R) mutant showed a modest reduction in γH2AX intensity compared to control neurons (Figure 35). These results indicate that acetylation of HDAC1 could modulate the cellular response to DSBs, with acetylation rendering neurons more susceptible, and deacetylation conferring resistance to genotoxic stress.

Because SIRT1 stimulates HDAC1 by deacetylating it, neurons expressing the acetyl-mimetic HDAC1K432Q mutant would be refractory to the effects of SIRT1 overexpression. To verify whether this was indeed the case, we treated neurons overexpressing SIRT1-flag together with either HDAC1-GFP or HDAC1K432Q-GFP, with etoposide and measured γH2AX intensity to assess their susceptibility to DSBs. Compared to controls, cells overexpressing either SIRT1 alone or SIRT1 together with HDAC1 showed significantly reduced γH2AX intensity upon etoposide treatment, indicating that SIRT1 confers resistance against DNA damaging agents (Figure 36). As before (Figure 35), neurons expressing the HDAC1K432Q mutant displayed increased γH2AX levels compared to controls. However, overexpression of SIRT1 had little effect on γH2AX intensity in neurons expressing the HDAC1K432Q mutant (Figure 36). Mutation of the lysine residue to glutamine had no effect on the ability of HDAC1 to bind SIRT1 (Figure 37). These results suggest that the neuroprotective effects of SIRT1 in response to DSBs require HDAC1 deacetylation.

Additionally, we again utilized the fluorescence-based NHEJ reporter system to understand how an interaction between SIRT1 and HDAC1 affects
DNA repair. We transfected the predigested NHEJ reporter construct in neurons expressing SIRT1 together with either a control shRNA or HDAC1 shRNA. Compared to controls expressing an empty vector, overexpression of SIRT1 was able to stimulate DNA repair through NHEJ, as indicated by the relative increase in GFP* cells (Figure 39). However, this increase was attenuated in neurons expressing HDAC1 shRNA (Figure 39), indicating that the stimulation of DNA repair by SIRT1 requires HDAC1. Similarly, we observed that overexpression of HDAC1 could stimulate NHEJ efficiency, but not in the absence of SIRT1 (Figure 39), and SIRT1 and HDAC1 also relied on each other to stimulate DNA repair through HR in proliferating U2OS cells (Figure 14). Together, our results suggest that an interaction between SIRT1 and HDAC1 is stimulated in response to DNA damage, which mutually stabilizes the two proteins at DSBs, and results in the deacetylation and activation of HDAC1. Furthermore, this cooperative relationship between SIRT1 and HDAC1 is essential for DSB signaling and DNA repair in neurons.

Based on the observations described above, we tested whether pharmacological activators of SIRT1 can confer neurons with increased resistance to DNA DSBs and how this relates to their ability to deacetylate HDAC1. We used a known activator of SIRT1, SRT3657 (Dai et al., 2010), and first determined that SRT3657 stimulates SIRT1 to deacetylate recombinant HDAC1 at K432 (Figure 12), but that it does not activate HDAC1 directly (Figure 38). We then incubated HEK293T cells with various concentrations of SRT3657 for 12 h and assessed HDAC1 acetylation at K432. The amount of acetylated HDAC1 decreased with increasing concentrations of SRT3657, indicating that activation of SIRT1 can stimulate HDAC1 deacetylation within cells (Figure 13). Following this, we treated cultured primary neurons with etoposide either in the absence or presence of SRT3657 and measured its effect on γH2AX intensity. In the presence of 10µM SRT3657, cultured neurons showed a dramatic reduction in γH2AX intensity compared to control neurons (Figure 15), in essence mimicking the effects of SIRT1 overexpression (Figure 36). However, this reduction in γH2AX intensity in the presence of SRT3657 was suppressed in
neurons expressing either the NTD of HDAC1 (which potentially titrates out the SIRT1-HDAC1 interaction), or the HDAC1K432Q mutant, again indicating that the protective effects of SRT3657 are dependent on the ability of SIRT1 to deacetylate HDAC1 (Figure 18).

Finally, to test how the ability of SRT3657 to activate SIRT1 and confer resistance to DSB formation affects neurodegeneration, we orally administered SRT3657 (30mg/kg) to CK-p25 mice and assessed γH2AX intensity and neuronal survival after 6 weeks of p25 induction. Six weeks of p25 induction resulted in marked neuronal loss and a pronounced increase in γH2AX intensity (Figure 19). Both neuronal loss and γH2AX intensity were significantly reduced in 6-week induced CK-p25 mice treated with SRT3657 (Figure 19). Because HDAC1 activity was shown to be downregulated in CK-p25 mice (Kim et al., 2008) and SRT3657 is able to stimulate HDAC1 deacetylation in vitro, we compared the levels of HDAC1 acetylation at K432 in CK-p25 mice after SRT3657 treatment. Compared to CK-p25 mice treated with vehicle, the levels of HDAC1 acetylated at K432 were sharply reduced in mice receiving SRT3657 treatment (Figure 20). These results suggest that activation of SIRT1 could suppress neurodegeneration by stimulating HDAC1, and highlight the therapeutic potential of SIRT1 activators in protecting neurons against genotoxic insults.
Discussion

The generation of DSBs triggers a signaling cascade that initiates with the activation of the ATM kinase, and recognition of the broken DNA ends by the MRE11 complex and by KU70/KU80. Our results, together with those from several other groups, place SIRT1 at the center of these early events (Figure 7). SIRT1 is recruited very rapidly to DSB sites ($t_{1/2} = 2.8$ s) (Figure 8) and exhibits a synergistic relationship with ATM. On the one hand, the localization of SIRT1 to DSBs is largely dependent on ATM activity (Figure 9). On the other hand, ATM autophosphorylation, ATM activity (at least in phosphorylating H2AX), and ATM stability at DSBs are significantly attenuated in the absence of SIRT1 (Figures 15, 20, 22 and 24). In addition, SIRT1 deacetylates NBS1 and promotes its phosphorylation by ATM (Yuan et al., 2007), and deacetylates and activates KU70 (Jeong et al., 2007), thereby facilitating DNA repair through NHEJ. Our current work has also revealed HDAC1, a Class I histone deacetylase, to be another important target of SIRT1 in the DSB response – an interaction between SIRT1 and HDAC1 is strengthened upon DNA damage, and SIRT1 deacetylates HDAC1 and stimulates its activity. Like SIRT1, HDAC1 also relocates rapidly to DSB sites in postmitotic neurons and its recruitment occurs as part of the NuRD complex, with NBS1 and KU70/KU80 also governing the dynamics of its localization (Figures 9 and 10). In addition, levels of HDAC1 at sites of DSBs are reduced in the absence of SIRT1 (Figures 30 and 31). These observations support the notion that HDAC1 is hierarchically downstream of SIRT1; however, the loss of HDAC1 also decreases the stability of ATM, NBS1, and SIRT1 at DSB sites (Figures 19, 30 and 31), indicating a more symbiotic relationship. One way in which HDAC1 could govern the stability of DSB signaling and repair complexes is through the modulation of chromatin configuration around DSBs. Although an “open” chromatin conformation allows repair proteins to access the damage sites more easily, recent studies suggest that a “closed” conformation in the initial stages after DSB formation could also favor DNA repair by retaining the
broken DNA ends in close proximity to each other and by repressing transcription in the vicinity of break sites (Ball and Yokomori, 2011; Miller et al., 2010). Together, these results allude to a more dynamic chromatin state at DNA damage sites. Recently, the acetylation of two lysine residues, H4K16 and H3K56, was shown to decrease in an HDAC1/HDAC2 dependent manner following DSB induction in U2OS cells (Miller et al., 2010). Furthermore, the inability to deacetylate these residues results in more relaxed chromatin and decreased DNA repair. Interestingly, while we verified the deacetylation of H4K16 following DSB induction in postmitotic neurons, we also observed that its deacetylation closely follows the deacetylation pattern of HDAC1 itself (Figure 33). In a previous report, modulation of HDAC1 activity through acetylation was shown to be important for the regulation of gene expression by steroid receptors (Qiu et al., 2006). We show for the first time that HDAC1 acetylation is also dynamically modulated following DSB induction in postmitotic neurons, with HDAC1 deacetylation occurring rapidly after DSB induction, followed by its increased acetylation at later times. However, HDAC1 acetylation in response to DSB induction is significantly increased in Sirt1 KO neurons (Figure 32). Moreover, an HDAC1 mutant mimicking a constitutively acetylated state for K432, showed an increased susceptibility to accumulate DSBs and was refractory to the normally protective effects of SIRT1 overexpression in the presence of DNA damaging agents (Figures 35 and 36). Based on these results, we favor a model in which SIRT1-mediated deacetylation of HDAC1 following DSB induction allows for the deacetylation of its histone targets, such as H4K16, which stabilizes DSB signaling components at damage sites and facilitates DNA repair through NHEJ (Chapter 4 Figure 1).
CHAPTER 4: Discussion and Future Directions
Introduction

Our genomes are constantly damaged by numerous endogenous and exogenous sources but cells must somehow maintain functionality in this arduous environment. This challenge is especially pronounced in non-proliferating cells such as neurons because once formed during development, neurons are retained for life. Mechanisms that preserve genomic integrity are critical for neural development and deficiencies in DNA repair manifest profoundly in the nervous system, resulting in microcephaly and progressive neurodegeneration. In addition, recent reports have suggested that unrepaired DNA damage could also underlie the cognitive decline associated with aging, and with neurodegeneration in Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS).

Of the lesions that threaten genomic integrity, double-strand breaks (DSBs) are considered to be particularly deleterious. In eukaryotic cells, DSBs elicit an elaborate signaling cascade, collectively called the DNA damage response (DDR), which includes pathways for DNA repair, transcriptional regulation, cell cycle arrest, and apoptosis. Diverse aspects of the DDR are coordinated by the multifunctional protein kinase, ATM, which is rapidly activated upon the induction of DSBs. For instance, ATM-mediated phosphorylation of the histone variant, H2AX (termed γH2AX), in the vicinity of DSBs is critical for activating the G2-M checkpoint, and for the stable retention of repair proteins at DSBs. Other well-established ATM substrates include NBS1, a component of the apical MRN (MRE11-RAD50-NBS1) complex that functions in the detection and repair of DSBs; the checkpoint kinase, CHK2; and p53, which itself plays multiple roles in DNA repair, checkpoint signaling, and apoptosis. DSBs are repaired using one of two pathways – nonhomologous end joining (NHEJ), which is error-prone and involves direct ligation of the broken DNA ends, and homologous recombination (HR), which is an error free pathway that is selectively utilized during S and G2 phases of the cell cycle.
In addition to phosphorylation, post-translational acetylation is also known to modulate the activity of a number of proteins. This highly reversible modification occurs on the ε-amino group of lysine and changes the electrostatic properties of proteins by neutralizing the positive charges on the amino group. Acetylation is regulated by the concerted actions of acetyltransferases and deacetylases, which add and remove acetyl groups, respectively. Many of these enzymes were originally identified as histone acetyltransferases (HATs) and histone deacetylases (HDACs) but were later found to possess activity towards non-histone proteins. Human cells contain at least 18 different HDACs that have been categorized into four classes: Class I HDACs, which comprise HDACs1-3 and HDAC8, resemble the yeast Rpd3; Class II HDACs, which consist of HDACs4-7, HDAC9, and HDAC10, show high similarity to the yeast Hda1; Class III NAD+-dependent HDACs (also referred to as sirtuins), constituting of SIRT1-7, show similarity to yeast Sir2; and Class IV HDACs, which are represented by a single member, HDAC11, shows some properties of both Class I and Class II HDACs. Observations from several groups implicate sirtuins and Class I HDACs in the DDR in proliferating cells. Both SIRT1 and its yeast ortholog, Sir2, relocate from their normal binding sites on repetitive DNA elements to sites of DNA DSBs and promote DNA repair. SIRT1 associates with and deacetylates NBS1, and maintains it in a hypoacetylated state, which is required for ionizing radiation-induced phosphorylation and activity of NBS1. Similarly, SIRT1 deacetylates Ku70 and the WRN protein, and stimulates their enzymatic activities in DNA repair, and deacetylates p53 to suppress apoptosis and promote cell survival. SIRT1 was also recently shown to deacetylate XPA and facilitate the repair of UV-induced DNA damage. In addition to SIRT1, human HDAC1 and HDAC2 were also shown to stimulate DSB repair through NHEJ, and to mediate the hypoacetylation of H3K56 and H4K16 following the induction of DSBs. HDAC1 was also shown to physically interact with ATM. However, the precise roles of these deacetylases in the DDR are not fully understood.

Although genomic integrity mechanisms have been a subject of intense investigation, much of our knowledge stems from studies conducted in
proliferating cells, and not postmitotic cells such as neurons. Previously, we reported that the levels of p25, a proteolytic cleavage fragment of the Cdk5 activating partner, p35, were induced by neuronal toxicity and elevated in brains undergoing neurodegeneration. Thereafter, we generated a bitransgenic mouse model (Ck-p25 mice) that allowed us to express p25 in the postnatal forebrain in an inducible manner and observed that this mouse model recapitulates various aspects of AD pathology, including the accumulation of amyloid-β peptides, neurofibrillary tau pathology, astrogliosis and neuronal death in the forebrain. However, two recent developments turned our focus towards the DNA damage response in neurons and its role in neurodegeneration – first, further characterization of CK-p25 mice revealed that the appearance of DNA DSBs and aberrant cell cycle re-entry by neurons precede all other neuropathological symptoms in these mice, raising the possibility that DNA damage could be an initiating event in the neurotoxicity caused by p25 generation. Second, we observed that overexpression of either SIRT1 or HDAC1 could prevent neuronal loss in CK-p25 mice. Based on these results, we decided to directly characterize whether SIRT1 and HDAC1 function in the neuronal DDR and investigate a potential relationship between their abilities to confer neuroprotection.

SIRT1 and HDAC1 are essential for the early steps of the DNA DSB response in neurons
The generation of DSBs triggers a signaling cascade that initiates with the activation of the ATM kinase, and recognition of the broken DNA ends by the MRE11 complex and by KU70/KU80. Our results, together with those from several other groups, place SIRT1 at the center of these early events (Figure 1). SIRT1 is recruited very rapidly to DSB sites ($\tau_{1/2} = 2.8$ s) (Chapter 3, Figure 8) and exhibits a synergistic relationship with ATM. On the one hand, the localization of SIRT1 to DSBs is largely dependent on ATM activity (Chapter 3, Figure 9). On the other hand, ATM autophosphorylation, ATM activity (at least in phosphorylating H2AX) and ATM stability at DSBs are significantly attenuated in
the absence of SIRT1 (Chapter 3, Figures 15, 20, 22 and 24). In addition, SIRT1 deacetylates NBS1 and promotes its phosphorylation by ATM (Yuan et al., 2007), and deacetylates and activates KU70 (Jeong et al., 2007), thereby facilitating DNA repair through NHEJ.

Figure 1. Model. The formation of DNA DSB triggers signaling cascade that includes the recognition of DNA ends by KU70/80 and/or the MRE11 complex, and activation of ATM, which phosphorylates its numerous targets (like histone H2AX) and coordinates various arms of the DSB response. SIRT1 function is crucial for some of these early events – SIRT1 is recruited rapidly to DSBs, where it performs a number of functions – it deacetylates and activates KU, deacetylates NBS1 and promotes its phosphorylation by ATM, and shares a synergistic relationship with ATM itself. Shortly after SIRT1 localization, HDAC1 is also recruited, most likely as part of the NuRD complex. This recruitment occurs in a KU70/80, NBS1, and SIRT1-dependent manner. HDAC1 then targets, such as H4K16Ac and H3K56Ac, whose deacetylation is essential for a more "closed" chromatin conformation and for DNA repair through NHEJ. HDAC1 activity in these processes is regulated by its own acetylation status, with HDAC1 deacetylation governing its activity. SIRT1 deacetylates HDAC1 and stimulates its activity. The result of this is the stabilization of DSB signaling and repair complexes at DNA damage sites that allows for efficient DSB repair.

Our current work has also revealed HDAC1, a Class I histone deacetylase, to be another important target of SIRT1 in the DSB response – an interaction between SIRT1 and HDAC1 is strengthened upon DNA damage, and
SIRT1 deacetylates HDAC1 and stimulates its activity. Like SIRT1, HDAC1 also relocates rapidly to DSB sites in postmitotic neurons and its recruitment occurs as part of the NuRD complex, with NBS1 and KU70/KU80 also governing the dynamics of its localization (Chapter 3, Figures 9 and 10). In addition, levels of HDAC1 at sites of DSBs are reduced in the absence of SIRT1 (Chapter 3, Figures 30 and 31). These observations support the notion that HDAC1 is hierarchically downstream of SIRT1; however, the loss of HDAC1 also decreases the stability of ATM, NBS1, and SIRT1 at DSB sites (Chapter 3, Figures 19, 30 and 31), indicating a more symbiotic relationship. One way in which HDAC1 could govern the stability of DSB signaling and repair complexes is through the modulation of chromatin configuration around DSBs. Although an “open” chromatin conformation allows repair proteins to access the damage sites more easily, recent studies suggest that a "closed" conformation in the initial stages after DSB formation could also favor DNA repair by retaining the broken DNA ends in close proximity to each other and by repressing transcription in the vicinity of break sites (Ball and Yokomori, 2011; Miller et al., 2010). Together, these results allude to a more dynamic chromatin state at DNA damage sites. Recently, the acetylation of two lysine residues, H4K16 and H3K56, was shown to decrease in an HDAC1/HDAC2 dependent manner following DSB induction in U2OS cells (Miller et al., 2010). Furthermore, the inability to deacetylate these residues results in more relaxed chromatin and decreased DNA repair. Interestingly, while we verified the deacetylation of H4K16 following DSB induction in postmitotic neurons, we also observed that its deacetylation closely follows the deacetylation pattern of HDAC1 itself (Chapter 3, Figure 33). In a previous report, modulation of HDAC1 activity through acetylation was shown to be important for the regulation of gene expression by steroid receptors (Qiu et al., 2006). We show for the first time that HDAC1 acetylation is also dynamically modulated following DSB induction in postmitotic neurons, with HDAC1 deacetylation occurring rapidly after DSB induction, followed by its increased acetylation at later times. However, HDAC1 acetylation in response to DSB induction is significantly increased in Sirt1 KO neurons (Chapter 3, Figure 32).
Moreover, an HDAC1 mutant mimicking a constitutively acetylated state for K432, showed an increased susceptibility to accumulate DSBs and was refractory to the normally protective effects of SIRT1 overexpression in the presence of DNA damaging agents (Chapter 3, Figures 35 and 36). Based on these results, we favor a model in which SIRT1-mediated deacetylation of HDAC1 following DSB induction allows for the deacetylation of its histone targets, such as H4K16, which stabilizes DSB signaling components at damage sites and facilitates DNA repair through NHEJ (Figure 1).

**Implications for neurodegenerative disorders**

We have focused our studies of HDAC1 and SIRT1 to the neuronal DDR. Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis pose a significant global health burden. The major risk factor in these disorders is age itself; however, the “age component” underlying this risk remains elusive (Yankner et al., 2008). Microarray analysis of postmortem brain samples from individuals of various ages revealed that genes encoding for crucial neural functions, including synaptic plasticity, learning, and memory, were downregulated after age 40, and this was associated with elevated levels of oxidative damage in the promoter regions of these genes (Lu et al., 2004). Concomitantly, stress response, antioxidant, and DNA repair genes were upregulated (Lu et al., 2004). Interestingly, SIRT1-target genes were overrepresented among age-upregulated genes in the mouse brain, and the relocalization of SIRT1 in response to chronic DNA damage was proposed to underlie some of these transcriptional changes (Oberdoerffer et al., 2008). In addition to this, DNA DSBs and an upregulation of DDR genes also correlated with the inhibition of HDAC1 activity in the CK-p25 mouse model, and these changes preceded neuronal loss and other neurotoxic effects in these mice that are also recapitulated in Alzheimer's disease (Adamec et al., 1999; Kim et al., 2008). Together, these results raise the interesting possibility that the accrual of DNA damage might be the “age component” that contributes critically to various neurological disorders, and underscores the importance of HDACs in conferring
neuroprotection. We propose that activators of SIRT1 could increase survival in at least two ways: (1) by directly stimulating SIRT1 activity and delaying the transcriptional changes caused by SIRT1 relocalization; and (2) indirectly, by stimulating SIRT1 to deacetylate and activate HDAC1, which in turn, protects neurons from genomic instability and death. A deeper understanding of these mechanisms could open new avenues for therapeutic intervention in neurodegenerative diseases.
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SIRT1 collaborates with ATM and HDAC1 to maintain genomic stability in neurons

Matthew M Dobbin1–3,9, Ram Madabhushi1–3,9, Ling Pan1–3, Yue Chen1,5, Dohoon Kim6, Jun Gao1–3,8, Biafra Ahanonu1–3, Ping-Chieh Pao1–3, Yi Qiu7, Yingming Zhao4,5 & Li-Huei Tsai1–3

Defects in DNA repair have been linked to cognitive decline with age and neurodegenerative disease, yet the mechanisms that protect neurons from genotoxic stress remain largely obscure. We sought to characterize the roles of the NAD+-dependent deacetylase SIRT1 in the neuronal response to DNA double-strand breaks (DSBs). We found that SIRT1 was rapidly recruited to DSBs in postmitotic neurons, where it showed a synergistic relationship with ataxia telangiectasia mutated (ATM). SIRT1 recruitment to breaks was ATM dependent; however, SIRT1 also stimulated ATM autophosphorylation and activity and stabilized ATM at DSB sites. After DSB induction, SIRT1 also bound the neuroprotective class I histone deacetylase HDAC1. We found that SIRT1 deacetylated HDAC1 and stimulated its enzymatic activity, which was necessary for DSB repair through the nonhomologous end-joining pathway. HDAC1 mutations that mimic a constitutively acetylated state rendered neurons more susceptible to DNA damage, whereas pharmacological SIRT1 activators that promoted HDAC1 deacetylation also reduced DNA damage in two mouse models of neurodegeneration. We propose that SIRT1 is an apical transducer of the DSB response and that SIRT1 activation offers an important therapeutic avenue in neurodegeneration.

RESULTS
SIRT1 is essential for DSB signaling and DNA repair in neurons
To determine whether SIRT1 is essential for genomic stability in neurons, we transduced neurons cultured from mouse embryos carryingloxP-flanked Sirt1 (Sirt1loxP/loxP) with a lentiviral vector carrying Cre recombinase tagged with enhanced GFP (Cre-eGFP) to delete Sirt1 (Supplementary Fig. 1a) and assessed DNA damage using a single-cell electrophoresis assay (comet assay)11. A significant fraction of Sirt1loxP/loxP neurons transduced with Cre-eGFP (hereafter referred to as Sirt1 knockout neurons) showed comet tails even without treatment with an exogenous DNA-damaging agent (Fig. 1a). In the presence of the DSB-inducing drug etoposide, Sirt1 knockout neurons had longer ‘tail moments’ compared to controls transduced with a vector carrying nonfunctional Cre (eGFP) (Fig. 1a). These results suggest that neurons lacking SIRT1 are more susceptible to DNA damage. In addition, whereas the tail moments in etoposide-treated control neurons were significantly reduced after recovery for 16 h, Sirt1 knockout neurons continued to have long comet tails, suggesting that Sirt1 knockout neurons are also deficient in DSB repair (Fig. 1a).

To verify this, we used a reporter assay system (Supplementary Fig. 1b,c)12 in which reconstitution of a functional GFP gene indicates successful DSB repair through the nonhomologous end-joining (NHEJ) pathway. In this assay, the number of GFP+ neurons transduced with Cre-eGFP was significantly reduced after Sirt1 knockout, confirming that SIRT1 is necessary for NHEJ-mediated DSB repair in neurons.
Notably, despite the elevated amounts of DNA damage (Fig. 1a), phosphorylation of H2AX (γH2AX) was reduced (by at least 30%) in Sirt1 knockout neurons that were challenged with etoposide (Fig. 1c). We obtained similar results in neurons pretreated with the SIRT1 inhibitor sirolin (Supplementary Fig. 1d), and together these findings suggest that initial events in DSB signaling could be disrupted in the absence of SIRT1 activity. To address this possibility, we first used the rare-cutting homing endonuclease I-PpoI to generate DSBs at defined loci within the genomes of primary Sirt1 knockout neurons transduced as in a. Top, I-PpoI cleavage sites in the Rna28s1 locus; bottom, results from ChIP experiments measuring the recruitment of phosphorylated ATM (pATM (Ser1981)) and NBS1 to cleavage sites within the Rna28s1 locus (*P < 0.05, Student’s t test). (e) Phosphorylation of ATM determined by western blotting in Sirt1 knockout neurons infected as in a and treated with either vehicle or 5 μM etoposide. Error bars (a,b,d), s.e.m.

ARGUMENTS

Figure 1 SIRT1 is necessary for initial DSB signaling events and DNA repair in neurons. (a) DNA damage assessed using a comet assay in Sirt1 knockout neurons infected with lentiviral vectors carrying either functional Cre recombinase (Cre-eGFP) or nonfunctional Cre (eGFP) that were treated with 5 μM etoposide (ETP) for 1 h and either allowed to recover (Rec.) for 16 h in the absence of etoposide or lysed immediately. Scale bar, 100 μm. The graph shows the comet tail moments (***P < 0.001, n = at least 50 neurons per condition, one-way analysis of variance (ANOVA)). AU, arbitrary units. (b) The number of GFP+ cells (indicating NHEJ-mediated repair) in cultured neurons that were transfected with a predigested NHEJ reporter construct (Supplementary Fig. 1b,c) together with either scrambled shRNA or SIRT1 shRNA (P < 0.05, unpaired t test). EBP, enhanced blue fluorescent protein. Scale bar, 20 μm. (c) Staining of Sirt1 knockout neurons using antibodies to γH2AX that were infected as in a and treated with either vehicle or 2 μM etoposide. Scale bar, 10 μm. (d) The synthetic, inducible system encoding the rare-cutting homing endonuclease, I-PpoI, that was used to generate DSBs at defined regions within the genomes of primary Sirt1 knockout neurons transduced as in a. Top, I-PpoI cleavage sites in the Rna28s1 locus; bottom, results from ChIP experiments measuring the recruitment of phosphorylated ATM (pATM (Ser1981)) and NBS1 to cleavage sites within the Rna28s1 locus (*P < 0.05, Student’s t test). (e) Phosphorylation of ATM determined by western blotting in Sirt1 knockout neurons infected as in a and treated with either vehicle or 5 μM etoposide. Error bars (a,b,d), s.e.m.

SIRT1 interacts physically with HDAC1

We previously showed that overexpression of the class 1 histone deacetylase, HDAC1, could also suppress neuronal loss during p25 expression. Because both SIRT1 and HDAC1 were able to suppress neuronal loss in the same mouse model, we reasoned that the two proteins might work collaboratively to promote genomic stability in neurons. Incubation of purified recombinant Flag-tagged HDAC1 (HDAC1-Flag) with recombinant histidine-tagged SIRT1 (SIRT1-His) followed by the precipitation of HDAC1 with anti-Flag–conjugated agarose beads coprecipitated SIRT1, suggesting a direct physical interaction between SIRT1 and HDAC1 (Fig. 2a). Notably, HDAC2, which shares ~85% similarity with HDAC1, was unable to bind SIRT1 under the same conditions (Fig. 2a). Next we generated and expressed multiple Flag-tagged fragments of HDAC1 (Fig. 2b) and mapped the interaction to the N-terminal domain (NTD) of HDAC1 (Fig. 2c). Notably, immunoprecipitation of endogenous HDAC1 indicated only weak binding with endogenous SIRT1 (Fig. 2d), but this interaction was considerably strengthened by induction of DNA damage (Fig. 2d).

Because an interaction between SIRT1 and HDAC1 is stimulated by DNA damage, we inquired whether SIRT1 and HDAC1 localize to sites of DNA DSBs in neurons. Co-localization analysis of cultured primary neurons revealed that SIRT1 and HDAC1 show a punctate distribution pattern that is devoid of chromocenters (subnuclear compartments consisting of densely packed chromatin) and nucleoli in mouse primary neurons. After treatment with etoposide, a substantial fraction of SIRT1 and HDAC1 colocalized with γH2AX foci (Fig. 2e), suggesting that both SIRT1 and HDAC1 are present at sites of DSBs in neurons. We next used laser microirradiation to generate subnuclear DSBs within the nuclei of individual Hoechst-stained primary neurons (Supplementary Fig. 2a,b). After microirradiation, both SIRT1 and HDAC1 showed strong enrichment within lesioned regions that were marked with γH2AX (Fig. 2f). In addition, we again used the I-PpoI system and targeted a unique I-PpoI cleavage site within the Dnahc7b locus on chromosome 1 for ChIP analysis. As described above (Fig. 1d), we detected a strong enrichment in phosphorylated ATM at chromatin proximal to the I-PpoI cleavage site after I-PpoI induction (Fig. 2g). The amounts of SIRT1 and HDAC1 were also enriched at damage-proximal chromatin after DSB generation, with pATM, SIRT1 and HDAC1 showing the strongest accumulation immediately 3’ to the cleavage site (Fig. 2g). Together these results suggest that SIRT1 and HDAC1 are recruited to sites of DNA DSBs in neurons.
Figure 2  SIRT1 and HDAC1 interact physically and localize to DSB sites in neurons. (a) The ability to retain SIRT1 in recombinant SIRT1-His that was incubated with either HDAC1-Flag or HDAC2-Flag, after which HDAC1 and HDAC2 were precipitated with anti Flag-conjugated agarose beads. (b) Diagram illustrating HDAC1 fragment constructs for interaction mapping. FL, full length; CTD, C-terminal domain; CAT, catalytic domain; aa, amino acid. (c) Immunoprecipitation and blotting with antibodies to Myc of the indicated Flag-tagged fragments expressed together with SIRT1-Myc. IP, immunoprecipitation; WB, western blotting. (d) Blotting with antibodies to SIRT1 of HT22 cells that were treated with camptothecin (CPT; 1 μM) and precipitated with antibodies to HDAC1. Ctl, control (before treatment with camptothecin). (e) Staining of etoposide-treated primary neurons with antibodies to either SIRT1 or HDAC1 and antibodies to γH2AX. Right, an intensity correlation analysis (ICA). Pixels from the input channel covarying positively with the corresponding signal from the γH2AX channel are indicated in yellow, and pixels covarying negatively are indicated in blue. Scale bars, 3 μm; insets, ×4 magnification. Veh, vehicle; DAPI, 4',6-diamidino-2-phenylindole. (f) Staining of primary neurons that were subjected to subnuclear, laser-generated DNA lesions with antibodies to either SIRT1 or HDAC1 and antibodies to γH2AX. Scale bar, 3 μm. (g) Recruitment of the indicated proteins at a unique cleavage site between exons 2 and 3 in DnaC7b assessed by CHIP after the rare-cutting homing endonuclease I-Ppol was used to generate DNA DSBs at defined genetic loci in mouse cortical neurons. Primers were designed at regular 1-kb intervals spanning 10 kb both 3' and 5' to the I-Ppol consensus site (red dashed line). Chr1, chromosome 1.

To further investigate the dynamics of SIRT1 and HDAC1 recruitment to DSB sites, we microirradiated Hoechst-stained primary neurons expressing either EmGFP-SIRT1 or HDAC1-EmGFP and monitored their localization to sites of laser-induced DSBs as a function of time. We detected an increase in EmGFP-SIRT1 accumulation in laser-lesioned regions almost immediately after DSB induction (τ1/2 = 3.21 ± 0.48 s (mean ± s.e.m.)) (Fig. 3a–c), whereas HDAC1-EmGFP accumulation became apparent by ~60 s (τ1/2 = 57.7 ± 5.8 s), indicating that SIRT1 localizes to DSB sites with faster kinetics than HDAC1. Next we individually knocked down various known DBS sensors and assessed their effects on SIRT1 and HDAC1 dynamics at laser-induced DSBs in live postmitotic neurons. The recruitment of SIRT1 to DSBs was strictly ATM dependent, and knockdown of ATM caused a marked reduction in both the maximal intensity and the kinetics of SIRT1 accrual at DSBs (Fig. 3d and Supplementary Fig. 2c). Thus, SIRT1 and ATM have a mutually dependent relationship, with SIRT1 being essential for ATM stability at DSBs and ATM activity after DSB induction (Fig. 1d,e) and ATM being necessary to recruit SIRT1 to DSBs. Conversely, the accumulation of HDAC1 was most severely affected by the knockdown of NBS1 and KU70 and KU80 (KU70/80) (Fig. 3d), with the latter also causing HDAC1 to accrue with slower kinetics (Supplementary Fig. 2e). Moreover, HDAC1 is probably recruited as part of the nucleosome remodeling and deacetylase (NuRD) complex, as knockdown of CHD4, an integral component of the NuRD complex, also severely disrupted HDAC1 localization to DSBs (Fig. 3d)16. Knockdown of individual DSB sensors had no effect on the expression of either EmGFP-SIRT1 or HDAC1-EmGFP (Supplementary Fig. 2d).

Overall, distinct proteins seemed to govern SIRT1 and HDAC1 recruitment to DSBs. However, because an interaction between SIRT1 and HDAC1 is enhanced by DNA damage and SIRT1 localizes to DSBs with faster kinetics than HDAC1, we tested the effect of SIRT1 knockdown on HDAC1 localization to laser-generated DSBs. In neurons transfected with SIRT1 short interfering RNAs (siRNAs), HDAC1 localization to laser-induced DSBs was markedly reduced (Fig. 3e), suggesting that in addition to the above factors, SIRT1 is also essential for HDAC1 recruitment. In corroboration with this finding, HDAC1 enrichment at I-Ppol-generated DSBs was also significantly diminished in Sirt1 knock-out neurons (Fig. 3f). Together these experiments describe the dynamics of SIRT1 and HDAC1 in response to DSB formation in living neurons, unveil a new synergistic relationship between SIRT1 and ATM that is crucial for signaling at DSBs and identify a new interaction between SIRT1 and HDAC1 that helps recruit HDAC1 to DSBs.
SIRT1 deacetylates HDAC1 and stimulates its enzymatic activity. Although an important function of the physical interaction between SIRT1 and HDAC1 could be to facilitate the recruitment of HDAC1 to DSB sites (Fig. 3a-f), HDAC1 is acetylated by the p300 acetyltransferase, and acetylation of HDAC1 inhibits its deacetylase activity. We therefore posited that the interaction of SIRT1 with HDAC1 might lead to the deacetylation and activation of HDAC1. Incubation of recombinant HDAC1 with increasing amounts of p300 recapitulated the previous observation that p300 acetylates HDAC1 (Supplementary Fig. 3a)\(^1\), whereas titration of SIRT1 in the HDAC1-p300 reactions decreased the acetylation of HDAC1 in a dose-dependent manner (Fig. 4a). In addition, whereas overexpression of HDAC1 together with p300 resulted in increased HDAC1 acetylation, coexpression of SIRT1 caused marked HDAC1 deacetylation (Fig. 4b), suggesting that SIRT1 can deacetylate HDAC1.

We next determined whether SIRT1-mediated deacetylation of HDAC1 affects its enzymatic activity. In a fluorescence-based reporter assay (Supplementary Fig. 3b), incubation of recombinant HDAC1 with p300 caused an approximate 40% reduction in HDAC1 activity, whereas its activity was stimulated by about 30% in the presence of SIRT1 (Fig. 4c). In addition, immunoprecipitated HDAC1 from Sirt1 knockout neurons showed a significant deficit in enzymatic activity compared to HDAC1 precipitated from control neurons (Fig. 4d). Conversely, SIRT1 overexpression resulted in stimulation of HDAC1 activity (Supplementary Fig. 3c). Together these results suggest that SIRT1-mediated deacetylation of HDAC1 stimulates its activity.

To specifically determine the residues within HDAC1 that are deacetylated by SIRT1, we incubated recombinant HDAC1 with either p300 alone or p300 and SIRT1 as described above and subjected the reaction mixtures to analysis by mass spectrometry. In the presence of p300 alone, HDAC1 was readily acetylated at residues Lys89, Lys220, Lys412, Lys432, Lys438, Lys439 and Lys441 (Fig. 4e-g). Label-free quantification indicated that the addition of SIRT1 resulted in decreased acetylation at all sites except Lys432 in a dose-dependent manner (Fig. 4h). An antibody that specifically recognizes acetylated Lys432 of HDAC1 (ref. 18) and quantitative western blotting further confirmed the ability of SIRT1 to deacetylate HDAC1 at this residue (Fig. 4i). Similarly, treatment of HEK293T cells with a pharmacological SIRT1 activator (compound #10)\(^1\) decreased HDAC1 acetylation at Lys432 in a dose-dependent manner (Fig. 4i). Furthermore, in a computationally predicted tertiary structure of HDAC1 (Supplementary Fig. 3d), the proximity of the region containing Lys432 to the NTD indicates that the binding of SIRT1 at the NTD could allow for deacetylation of residues at the C terminus.
Figure 4 SIRT1 deacetylates HDAC1 at residue Lys432 and stimulates its enzymatic activity. (a) HDAC1 acetylation assessed by western blotting of recombinant SIRT1 that was titrated over a fixed amount of p300 and HDAC1 (*P < 0.05, n = 3, one-way ANOVA). KAc, acetylated lysine. (b) Immunoprecipitation and acetylation status of HDAC1 (probed using an antibody to acetylated lysine) in HT22 cells expressing HDAC1-Flag, hemagglutinin-tagged p300 (p300-HA) or SIRT1-Myc. (c) Enzymatic activity of HDAC1 that was preincubated with either p300 or SIRT1, as in a and b, assessed using a fluorescence-based HDAC enzymatic activity assay (Supplementary Fig. 3b) (**P < 0.01, n = 3, one-way ANOVA). (d) Immunoprecipitation and enzymatic activity of HDAC1, as in c, in Sirt1lox/lox neurons that were infected with Cre-eGFP and eGFP lentiviral vectors (*P < 0.05, n = 3, unpaired t-test). (e) Diagram depicting the acetylated lysine residues in HDAC1 and HDAC2. (f) Annotated tandem mass spectrometry spectrum of the lysine-acetylated peptide NSSNFKAKAR (where subscript ac denotes acetylation) that identified lysine acetylation sites at Lys432, Lys439 and Lys441 of HDAC1 after reaction with p300. The b and y ions represent collision-induced peptide fragment ions containing the N or C terminal, respectively. The asterisks indicate fragment ions with neutral loss of amine. (g) Label-free quantification for each lysine acetylation site using a protein abundance-normalized peptide-precursor ion intensity showing that lysine acetylation abundance on p300-treated HDAC1 decreased after the addition of SIRT1 to the reaction. Ac, acetylation of the indicated lysine. (h) Acetylation of HDAC1, as in a, of recombinant SIRT1 that was incubated together with p300 and HDAC1 assessed using an antibody specific to acetylated Lys432. The arrowheads indicate nonspecific cross-reacting bands in the lanes containing recombinant SIRT1 (*P < 0.05, n = 3, unpaired t-test). (i) HDAC1 acetylation at Lys432 in HEK293T cells that were treated with the indicated concentrations of the SIRT1 activator compound #10 for 12 h. Error bars (a,c,d,h,i, s.e.m.

Of all the acetylatable residues in HDAC1, Lys432 has been shown to be particularly important for its enzymatic activity, and mutation of this residue to an acetyl mimetic glutamine (K432Q) almost completely abolished HDAC1 activity. Moreover, whereas the remaining lysines are conserved between HDAC1 and the closely related HDAC2, Lys432 in HDAC1 is occupied instead by arginine in HDAC2 (Fig. 4e), and HDAC1, but not HDAC2, can be acetylated by p300 (refs. 17,20). Taken together these results suggest that in addition to facilitating the recruitment of HDAC1 to DSB sites, SIRT1 also exists in an enzyme-substrate relationship with HDAC1 wherein SIRT1 deacetylates HDAC1 at a crucial lysine residue, Lys432, thereby stimulating its enzymatic activity.
**Figure 5** Deacetylation of HDAC1 is essential for DSB repair in neurons. (a) DNA damage assessed using comet assay in HDac1<sup>+/-</sup> embryos that were infected as in Figure 1a and treated with etoposide (**P < 0.01, n = at least 50 neurons per condition, one-way ANOVA). Scale bar, 100 μm. (b) HDAC acetylation at Lys432 assessed by western probing of primary neurons cultured from Sirt1<sup>lox/lox</sup> embryos that were infected as in Figure 1a and treated with 5 μM etoposide for 2 h (**P < 0.05, n = 3 independent experiments, one-way ANOVA). (c) Western blot analysis probing the indicated acetylation marks in cultured primary neurons (7 d in vitro) that were treated with 5 μM etoposide for 30 min, after which the cells were lysed either immediately or after recovery from etoposide washout for the indicated times and electrophoresed. Untr. untreated. (d) Staining with antibodies to γH2AX of primary neurons that were transfected with the indicated vectors and treated with etoposide (2 μM) for 1 h. Scale bar, 10 μm (**P < 0.05, +++P < 0.001, n = at least 25 neurons per condition and 4 independent experiments, one-way ANOVA). (e) As in d but using cultured primary neurons expressing SIRT1-Flag together with either HDAC1-EmGFP or HDAC1<sup>K432Q</sup>-EmGFP that were treated with etoposide (2 μM) for 1 h. Scale bar, 15 μm. The quantification is shown to the right (**P < 0.05, n = at least 25 neurons per condition and 3 independent experiments, one-way ANOVA). (f) The number of GFP<sup>+</sup> cells (assessed as a measure of DNA repair using NHEJ) of cultured primary neurons expressing either SIRT1 together with HDAC1 shRNA that were transfected with the predestigned NHEJ reporter construct (**P < 0.05, n = 3 independent experiments, one-way ANOVA). Error bars (a,b,d-f), s.e.m.

**HDAC1 deacetylation by SIRT1**

To understand whether HDAC1 deacetylation has a role in DSB signaling and repair in neurons, we characterized the effects of HDAC1 loss in these processes. In comet assays, *Hdac1* knockout neurons had longer tail moments than controls and, similarly to *Sirt1* knockout neurons (Fig. 1a), were unable to recover from etoposide-induced DSBs (Fig. 5a). This suggests that neurons become more susceptible to DSBs in the absence of HDAC1 and that HDAC1 is essential for DSB repair in neurons. In contrast to *Sirt1*, however, HDAC1 had no effect on ATM autophosphorylation (Supplementary Fig. 4a), and γH2AX intensity was increased in *Hdac1* knockout neurons compared to controls after etoposide treatment (Supplementary Fig. 4b). We obtained similar results in cultured primary neurons expressing a catalytically inactive HDAC1, HDAC1<sup>H141Y</sup> (Supplementary Fig. 4c). Thus, although HDAC1 is essential for DNA repair in neurons, initial events in DSB signaling such as ATM and H2AX phosphorylation do not require HDAC1 activity. These results are consistent with the notion that HDAC1 functions downstream of *Sirt1* in the DSB response.

We next assessed the status of HDAC1 acetylation in *Sirt1* knockout neurons after etoposide treatment. Whereas HDAC1 acetylation at Lys432 was increased in etoposide-treated control neurons, this increase was far more pronounced in *Sirt1* knockout neurons (Fig. 5b), suggesting that the acetylation of HDAC1 is modulated in response to DSB formation and that SIRT1 maintains HDAC1 in a deacetylated and active state in neurons. However, given that HDAC1 is essential for DSB repair, we found it peculiar that the acetylation of HDAC1 is elevated after DSB formation. To further clarify this matter, we briefly treated cultured primary neurons with etoposide (30 min) followed by washout and recovery in etoposide-free medium. We then prepared lysates at hourly intervals and monitored HDAC1 acetylation at Lys432 as a function of time after etoposide treatment. Notably, compared to untreated controls, neurons treated with etoposide for 30 min showed a reduction in Lys432 acetylation (Fig. 5c). This trend continued until 1 h after etoposide washout, after which time the amount of acetylated Lys432 began to rise again, surpassing the amount of acetylation in untreated controls by about 4 h after washout (Fig. 5c). Furthermore, this pattern of HDAC1 acetylation mirrored changes in the acetylation of histone H4 Lys16 (H4K16) (Fig. 5c), a previously identified HDAC1 target<sup>1</sup>, and the amount of H4K16 acetylation was increased in *Hdac1* knockout neurons (Supplementary Fig. 4d).

To further understand the importance of HDAC1 deacetylation in the DSB response, we overexpressed eGFP-tagged variants of HDAC1 carrying either a K432Q (acetylation mimetic) or a K432R (acetylation resistant) mutation in cultured primary neurons. In control neurons expressing eGFP alone, etoposide treatment readily triggered the formation of γH2AX foci, and consistent with previous observations, overexpression of HDAC1-EmGFP caused a reduction in the number of γH2AX foci (Fig. 5d). In contrast, neurons expressing the K432Q mutant showed γH2AX foci even in the absence of etoposide treatment and a substantial increase in the number of foci in the presence of etoposide (Fig. 5d). Conversely, neurons expressing the K432R mutant had a modest reduction in γH2AX intensity compared to controls, indicating that constitutive acetylation of HDAC1 renders neurons more susceptible to genotoxic insults, especially DSBs.

Because SIRT1 stimulates HDAC1 through deacetylation, we predicted that the acetyl-mimetic HDAC1<sup>K432Q</sup> mutant would also be refractory to the effects of SIRT1 overexpression. In the presence of etoposide, neurons overexpressing SIRT1 showed a significant
Figure 6 Pharmacological SIRT1 activation can protect neurons against DNA damage in vivo. (a) Acetylation of HDAC1 at Lys432 assessed by quantitative western blotting in brain lysates of CK-p25 mice that expressed the p25 transgene for 6 weeks and were administered either vehicle or 30 mg per kg body weight of compound #10. (b) Representative immunohistochemical images showing γH2AX and NeuN staining in CK-p25 mice that expressed the p25 transgene for 6 weeks and were administered either compound #10 or vehicle (Online Methods). Scale bar, 100 μm. (c) HDAC1 acetylation at Lys432 assessed by western blotting of hippocampal lysates from 2-month-old tau P301S transgenic mice that were treated with SIRT1 activator (d) Brain sections from 2-month-old P301S transgenic (Tg) mice that were administered either vehicle or 30 mg per kg body weight of compound #10 through oral gavage (5 mice per group) once daily for 2 weeks stained with antibodies to γH2AX and NeuN. Scale bar, 100 μm.

reduction in the number of γH2AX foci compared to controls (data not shown); however, SIRT1 overexpression had little effect on γH2AX intensity in neurons also expressing the HDAC1 K432Q mutant (Fig. 5e). Similarly to SIRT1 overexpression, treatment of neurons with the pharmacological SIRT1 activator also caused a reduction in γH2AX intensity (Supplementary Fig. 4e,f), and neurons expressing the K432Q and K432R mutants were also refractory to a SIRT1 activator-mediated reduction in γH2AX intensity (Supplementary Fig. 4f). Mutation of the lysine residue to glutamine had no effect on the ability of HDAC1 to bind SIRT1 (Supplementary Fig. 4g). However, expression of the NTD fragment of HDAC1, which would compete with endogenous HDAC1 for SIRT1 binding, resulted in increased γH2AX intensity compared to controls (Supplementary Fig. 4f). Also, SIRT1 activator treatment could not stimulate the activity of purified recombinant HDAC1 directly in the absence of SIRT1 (Supplementary Fig. 4g). We again used the fluorescence-based NHEJ reporter system wherein we transfected the pre-digested reporter construct in neurons expressing SIRT1 together with either a control short hairpin RNA (shRNA) or HDAC1 shRNA. SIRT1 activator overexpression in neurons stimulated NHEJ-mediated DSB repair, as indicated by an increase in GFP+ cells compared controls (Fig. 5f). However, this increase was attenuated in neurons expressing HDAC1 shRNA (Fig. 5f). Taken together these results suggest that the ability of SIRT1 to protect against DNA damage and stimulate DNA repair requires it to interact with and deacetylate HDAC1.

On the basis of these observations, we assessed whether SIRT1 activation also affects the number of DSBs and HDAC1 acetylation in neurodegenerating CK-p25 mice by orally administering the pharmacological SIRT1 activator to these mice. Western blot analysis of hippocampal lysates after 6 weeks of p25 induction revealed a sharp reduction in HDAC1 Lys432 acetylation in the CK-p25 mice that we treated with the SIRT1 activator compared to CK-p25 mice treated with a vehicle control (Fig. 6a). In addition, whereas vehicle-treated CK-p25 mice had a marked increase in the number of γH2AX-positive cells in the hippocampus, the number of γH2AX-positive cells was reduced by about 40% in CK-p25 mice administered the SIRT1 activator (Fig. 6b). These results suggest a strong correlation between SIRT1 activation, HDAC1 deacetylation and a reduction in the number of DNA DSBs. Additionally, administration of the SIRT1 activator was also able to reduce HDAC1 acetylation and γH2AX intensity in 2-month-old human tau transgenic (P301S) mice (Fig. 6c,d). Together these results highlight the therapeutic potential of SIRT1 activators against neurodegeneration.

DISCUSSION Overall our data suggest that SIRT1 primes the cellular response to DNA DSBs by stimulating the activities of ATM and HDAC1. After being recruited to DSBs in an ATM-dependent manner, SIRT1 in turn stimulates ATM autophosphorylation and ATM recruitment to DSB sites and thereby primes the cellular DSB signaling cascade (Supplementary Fig. 5). After DSB induction, the MRE11-RAD50-NBS1 complex is known to activate ATM through an interaction between NBS1 and ATM23. SIRT1 also interacts with and deacetylates NBS1 (ref. 24), and NBS1 recruitment to DSBs is compromised in Sirt1 knockout neurons (Fig. 1d). It is therefore intriguing to consider the SIRT1-NBS1 interaction as a potential mechanism of ATM activation. However, ATM is also known to be an acetylated protein25, and an equally interesting possibility involves SIRT1-mediated deacetylation of ATM being important for ATM autophosphorylation and activity.

In addition to activating ATM, SIRT1 participates in conjunction with Ku70/80, NBS1 and the NuRD complex to stabilize HDAC1 at DSB sites. Furthermore, SIRT1 deacetylase and activates HDAC1, thus facilitating the dynamic regulation of HDAC1 activity that is essential for DSB repair through NHEJ. Our data are consistent with the notion that HDAC1 functions in DNA repair by affecting chromatin configuration through epigenetic modification. For instance, acetylation of H4K16 and H3K36 decrease in an HDAC1- and HDAC2-dependent manner after DSB induction, and the inability to deacetylate these residues results in more relaxed chromatin and decreased amounts of DNA repair21,26. However, an 'open' chromatin configuration is also important for repair because it allows repair proteins to access damaged sites easily26. The 'closed' chromatin configuration (probably mediated by HDAC1 and other proteins) in the initial stages after DSB formation could allow for the broken DNA ends to be retained in close proximity and for transcriptional silencing in their vicinity, after which control of 'opening' of the chromatin could grant access to repair and signaling factors. The dynamic modulation of HDAC1 activity through its acetylation and the biphasic pattern of H4K16 acetylation (Fig. 5e)21 are consistent with such a model.
here that one arm of such modulation is conferred by SIRT1, which deacetylates and activates HDAC1. The other arm, probably involving p300, has yet to be unraveled. Several histone acetyltransferases (HATs), including p300, HMGA and Tip60, are known to function in the DNA DSB response1,2. Moreover, these HATs are also deacetylated by SIRT1, and SIRT1-mediated deacetylation has been shown to inhibit their HAT activity3,4. The importance of these interactions in chromatin organization and signaling at DSBs is only beginning to be unraveled, and although our work emphasizes the role of the SIRT1-HDAC1 connection in DSB repair, the relative importance of SIRT1-mediated deacetylation of the above-mentioned substrates warrants further investigation.

In neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, the major risk factor is age itself5,6. Microarray analysis of postmortem human brain samples has revealed that genes encoding for synaptic transmission, learning and memory are downregulated after age 45, and this is associated with elevated amounts of oxidative damage in the promoters of the downregulated genes1. In addition, DNA DSBs and an upregulation of DNA damage-response genes precede the emergence of all other Alzheimer's disease-like neuropathological hallmarks in CK-p25 mice7,8, and elevated amounts of DNA strand breaks have been observed in the Alzheimer's disease brain9. Together these results raise the possibility that the accrual of DNA damage with age could underlie the pathological changes that are associated with neurodegenerative disease. SIRT1 is known to directly modulate synaptic plasticity and memory formation9,10, and SIRT1 redistribution in response to chronic DNA damage is thought to underlie some of the transcriptional changes in the aging brain9. Considering these observations, the benefits conferred by pharmacological activation of SIRT1 could be substantial.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

This study was designed by M.M.D., R.M. and L.-H.T. and was directed and coordinated by L.-H.T. M.M.D and R.M. planned and performed most of the experiments. L.P. maintained the Sirt1<sup>fl/fl</sup>MplR<sup>Cre</sup> and HNk<sup>fl/fl</sup>MplR<sup>Cre</sup> mice and, together with B.A., helped with the microirradiation experiments. Y.C. and Y.Z conducted the mass spectrometry analysis of HDAC1 acetylation. D.K. and J.G performed some preliminary experiments with CK-p25 mice, and J.G performed the compound 10 treatment and subsequent analysis of CK-p25 mice. F.C.P. contributed to statistical analysis and quantification of several experiments, and Y.Q. developed and provided the antibody to acetylated HDAC1 1lys432. R.M., M.M.D. and L.-H.T. wrote the manuscript with critical input from all the authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
ONLINE METHODS

Mouse strains, expression constructs, shRNA constructs and virus generation. All mouse work was approved by the Committee for Animal Care of the Division of Comparative Medicine at MIT. HDAC1 pmouse and SirT1 pmouse were the kind gifts of E.N. Olson and F.W. Alt, respectively, and were as described.33,34 Mouse HDAC1 and SirT1 were subcloned into the pcDNA6.2/CMV-EmGFP Gateway Vector (Invitrogen, V350-20) and the pcDNA6.2/N-EmGFP Gateway Vector (Invitrogen, V350-20), resulting in C-terminal and N-terminal fusion proteins, respectively. HDAC1 fragments were constructed according to functional protein domains as determined by bioinformatic analysis with Pfam and as described previously.27 The Stratagene QuickChange Site-Directed Mutagenesis Kit (Stratagene, 200518) was used to generate HDAC1 mutants mimicking either a constitutively acetylated or nonacetylatable state at amino acid position 432 (K432Q and K432R, respectively). Because fusion of several different affinity tags to the N terminus of HDAC1 has been shown to interfere with its catalytic activity, all HDAC1 fusion constructs were generated as C-terminal fusions. HDAC1 and SirT1 shRNA constructs and catalytic residue mutants were as previously described.28,29 The HA–ER1–PpoI overexpression construct was obtained from the lab of M. Kastan (St. Jude's Children's Research Hospital) and was modified for the production of lentiviruses by subcloning into a lentiviral backbone containing a T2A–red fluorescent protein (RFP) under the control of the PGK promoter. Lentiviral constructs, lent-cre and lenti-cre, were the kind gift of R. Huganir (Johns Hopkins University) and were as reported previously.36 Pooled siRNA oligonucleotides targeting mouse SirT1 (sc-29344), ATM (sc-29762), MRE11 (sc-37396), NBS1 (sc-36062) and RAD50 (sc-37398) and control scrambled siRNA (sc-37397) were obtained from Santa Cruz Biotechnologies. Pooled siRNA oligonucleotides targeting mouse Ku70 (EMU067171) and Ku80 (EMU05221) were obtained from Sigma-Aldrich. Pooled siRNA oligonucleotides targeting mouse Chd4 (L-052142-00-0005) were obtained from Dharmacon. Antibodies. HDAC1 antibodies to acetylated HDAC1 (Ly532Ac) were generated from rabbits injected with an acetylated C-terminal peptide (peptide sequence GEERGRKG, NSNF). The antibodies used for staining were as follows: anti-HDAC1 (Abcam, ab15028, 1:1,000), anti-SirT1 (Abcam, ab7911, 1:1,000, http://www.abcam.com/sirt1-antibody-ab7911.html), anti-pATM (Ser1981) (Abcam, ab36810, 1:1,000, http://www.abcam.com/HDAC1-antibody-1T9-Chip-Grade-ab31263.html), anti-SIRTI (Abcam, ab7343, 1:1,000, http://www.abcam.com/sirtl-antibody-ab31263.html), anti-yH2AX (Millipore, antibody-1T9-ChIP-Grade-ab31263.html), anti-SIRTL (Abcam, ab7343, 1:1,000, http://www.abcam.com/sirtl-antibody-ab7343.html), and anti-HA (Millipore, ab3254, 1:1,000, http://www.millipore.com/catalogue/item/05-515, 1:1,000, http://www.millipore.com/catalogue/item/05-515, anti-HA (Millipore, ab3254, 1:1,000, http://www.millipore.com/catalogue/item/05-515, 1:1,000, ref. 18), anti-pATM (Ser1981) (Abcam, ab36810, 1:1,000, http://www.abcam.com/HDAC1-antibody-1T9-Chip-Grade-ab31263.html) and anti-ATM (Abcam, ab2618, 1:1,000, http://www.abcam.com/ATM-antibody-5c2-ab2618.html). Antibodies used for ChIP and immunoprecipitation experiments were all used at an amount of 2 μg per reaction and were as follows: anti-histone H4, pan (Millipore, 04-858, http://www.millipore.com/catalogue/item/04-858, anti-pATM (Ser1981) (Abcam, ab36810, 1:1,000, http://www.abcam.com/ATM-antibody-5c2-ab2618.html).

For primary neurons, dissociated cortical neurons dissected from embryonic day 16–18 Swiss-Webster mice were plated at a density of 500,000 cells per well in 24-well plates, 2 million cells per plate in 35-mm glass bottom plates and 15 million cells per plate in 10-cm plates. The plates were coated beforehand by incubation with poly-ν-l-lysine (0.05 mg ml⁻¹) and laminin (0.005 mg ml⁻¹) for 1 h at 37 °C followed by washing twice with H₂O. Neurons were maintained in neurobasal medium (Gibco, 21103) supplemented with l-glutamine (5 mM), penicillin and streptomycin and B27 neuronal additive. Phenol red–free neurobasal medium (Gibco, 12348) was used as a substitute for the neuron cultures used in live-imaging experiments. Cell lines and primary neuron cultures were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, 11668) for at least 1 h in medium lacking antibiotics, after which cells were washed in warmed medium and given at least 24 h to allow for construct expression before usage. For live-imaging experiments using siRNA, 0.75 μg siRNA was cotransfected with either HDAC1-EmGFP or EmGFP-SirT1 and given 48 h to allow for sufficient knockdown before imaging. For viral gene transduction, the virus was added directly to the culture medium. I-PpoI-ER was induced by adding 4-hydroxystrophanthin (Sigma, H7904) to a final concentration of 1 μM and incubated for 6 h before fixation.

Western blotting and immunoprecipitation. For both primary neurons and cell lines, 1.5–2 × 10⁶ cells were washed once with PBS and lysed for 10 min on ice in RIPA buffer (150 mM NaCl, 1% IGEPA, 0.5% NaDod, 0.1% SDS and 50 mM Tris, pH 8.0, supplemented with protease inhibitors) on the plates in which they were originally grown. Cells were then collected by scraping and were rotated for 30 min at 4 °C. Supernatant was collected after centrifugation (13,000 r.p.m., 10 min, 4 °C). For each sample, SDS protein loading buffer was added to 1x and boiled (95 °C, 10 min) before loading onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Gels were transferred to polyvinylidene fluoride membranes (200 mA constant current) and blocked with 3% bovine serum albumin (BSA) in PBS plus TWEEN-20 (PBS-T) for 1 h before application of primary antibodies. Membranes were visualized with either electrochemiluminescence and autoradiographic film detection or the LiCor Odyssey quantitative western imaging system.

For immunoprecipitations, 1 mg of total protein lysate was used for each condition and brought to a total volume of 500 μl with RIPA buffer. The appropriate antibody was then added, and the mixture was incubated on a rotator overnight at 4 °C. As required, protein A/G-conjugated agarose beads (GE Health Science, 17-5280/17-0618) or Flag M2 affinity gel (Sigma, A2220) were equilibrated with RIPA buffer, blocked overnight with 3% BSA and washed before adding to the samples. For all immunoprecipitations, a total volume of 30 μl of bead slurry was used per reaction mix. The reaction mixtures were incubated for 1 h at 4 °C after which they were washed four times and denatured by boiling (95 °C, 10 min) in RIPA buffer containing 1x SDS sample buffer.

Immunostaining, image acquisition and analysis. For immunostaining, cells were fixed by incubating with 4% paraformaldehyde for 10 min at room temperature and incubated with blocking buffer (5% normal donkey serum and 0.3% Triton X-100 in PBS) for 1 h. Primary antibodies were diluted in blocking buffer and incubated with cells overnight at 4 °C. Primary antibodies were visualized using the appropriate secondary antibodies conjugated to Cy2, Cy3 and Cy5 fluorescent dyes (Jackson ImmunoResearch Laboratories Inc., 1:500, 711-545-152, 711-165-152, 715-485-150, 715-165-150). Images for all fixed cells were acquired on a Zeiss LSM510 laser-scanning confocal microscope and subsequently deconvolved using theoretical point spread functions generated in ImageJ along with the Tikhonov-Miller iterative image restoration algorithm, which was implemented in the DeconvolutionLab plug-in written for ImageJ. To assess and analyze images in a quantitative and unbiased manner, CellProfiler automated image analysis software was trained to measure the per-cell H2AX signal from a minimum of 100 cells per condition for all imaging experiments. For experiments using tagged proteins, CellProfiler was trained to consider transfected cells exclusively for quantification. Coloocalization was assessed using ICA computation, which highlights pixels either negatively or positively covarying between two input channels. A plug in implementing this technique has been written as a plug-in for ImageJ and is freely available for download (http://imagej.nih.gov/ij/plugins/mbf/).
Image processing and analysis for microirradiation live imaging. All data acquired from microirradiation time lapses were processed according to the iterative deconvolution strategy described above before analysis. Normalized relative fluorescence ($F_{rel}$) within the 2-μm rectangular-strip ROI was quantified for each time point using a computation that compensates for both background signal and fluorescence loss due to observational bleaching. $F_{rel}$ values were extracted from $F_{rel}$ values by first converting them into fractional fluorescence values as described previously, and Prism5 was then used to plot and fit curves to this data.

**Liquid chromatography—mass spectrometry analysis.** Gel bands containing HDAC1 were excised and in-gel digested using a protocol previously described. The tryptic peptides were solubilized in high-performance liquid chromatography (HPLC) buffer A (0.1% formic acid in water) and loaded onto a capillary HPLC column (10 cm, 75 μm inside diameter) self-packaged with Jupiter C12 resin (Phenomenex). Samples were separated using a 90-min linear gradient of 5-30% HPLC buffer B (0.1% formic acid in acetonitrile) and analysed by an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific Inc.). The tandem mass spectrometry data were analyzed with the Mascot search engine (Matrix Science, v2.1) with a Mascot cutoff score of 10 and P < 0.05 and with subsequent manual verification. Label-free quantification was performed for each lysine acetylation site on the basis of peptide precursor ion intensity normalized by protein abundance ratios.

**Statistical methods.** Standard statistical methods consistent with previous reports using similar assays, techniques and methods were used to analyze all data. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications. Analysis of $\gamma$H2AX intensity in CK-25 and tau P301S mice after treatment with SIRT1 activator were performed blind to the conditions of the experiment. For most other analyses, data collection and analysis were not performed blind to experimental conditions. Data were collected and processed randomly and appropriately blanked. The data distributions were assumed to be normal, but this was not formally tested.

Corrigendum: SIRT1 collaborates with ATM and HDAC1 to maintain genomic stability in neurons

Matthew M Dobbin, Ram Madabhushi, Ling Pan, Yue Chen, Dohoon Kim, Jun Gao, Biafra Ahononu, Ping-Chieh Pao, Yi Qiu, Yingming Zhao & Li-Huei Tsai

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In the version of this article initially published online, author Biafra Ahononu’s name was misspelled Ahononu. The error has been corrected for the print, PDF and HTML versions of this article.
Supplementary Figure 1. a, Schematic for generating Sirt1 KO neurons. Dissociated primary cortical neurons were cultured from E16 Sirt1 F/F embryos. DIV3 cultures were infected with a lentiviral vector carrying either a functional Cre recombinase (Cre-eGFP) or a non-functional Cre (eGFP). Neurons were usually used 7 days after infection with Cre-eGFP. A similar strategy was used to generate Hdac1 KO neurons. b, Construct used to measure efficiency of DNA repair using NHEJ. In this construct, a functional eGFP gene is interrupted by an intron. The presence of an adenoviral exon sequence within the intron prevents it from normally being spliced out. Generation of a DSB using HindIII and its subsequent repair using NHEJ disrupts the adenoviral exon sequence. Consequently, the intron is spliced out, allowing for expression of GFP. In this way, GFP+ cells can be used to score the efficiency of NHEJ. c, Outline of the experimental scheme used to adapt the NHEJ reporter assay to cultured primary neurons in our study. d, Primary cortical neurons (DIV14) were incubated overnight with the SIRT1 inhibitor, sirtinol (20μM final), then treated with 2μM etoposide for 1 h and analyzed as in Fig. 1c. Scale bar = 10μm.
Supplementary Figure 2. a, Schematic of laser microirradiation. A Zeiss LSM710 inverted laser scanning confocal microscope equipped with a 405 nm diode laser was used to irradiate a thin sub-nuclear strip of Hoechst-stained primary neurons. Localization of proteins to sites of laser-induced DNA DSBs can be monitored as increased fluorescence intensity within lesioned regions as visualized either by immunocytochemistry of fixed cells (for instance, γH2AX) or through live imaging of cells carrying fluorescently tagged repair proteins. b, Serial attenuation of transmitted 405 nm wavelength light emitted from a continuous-wave diode laser yields a dose-dependency in γH2AX signal intensity within lesion ROIs. ROI area (2 μm²), laser power (100%), and scan iterations were held constant for Hoechst33242 pre-sensitized neuronal nuclei, while percent transmission was varied as indicated. Neurons were then fixed and stained with γH2AX. Scale bar = 7 μM. c, Table indicating the time taken by EmGFP-SIRT1 (left) and HDAC1-EmGFP (right) to attain half-maximal fluorescence intensity in the lesioned region following the knockdown of the DSB components in Fig. 3d. d, Representative images of EmGFP-SIRT1 (top) and HDAC1-EmGFP (bottom) expressing neurons that were transfected with the indicated siRNAs to show that the various siRNAs did not affect expression of the two proteins.

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Supplementary Figure 3

Supplementary Figure 3, a, Increasing amounts of p300 were incubated with a fixed amount of HDAC1 and the effect of p300 on the acetylation of HDAC1 was assessed using quantitative western blotting (* p < 0.05, one-way ANOVA). b, Schematic of a fluorescence-based reporter assay used to measure HDAC1 enzymatic activity. Deacetylation of the substrate sensitizes it to cleavage by trypsin, which results in the release of a fluorescent moiety (green). Fluorescence intensity is thus used as an indicator of deacetylase activity. c, HEK293T cells were transfected with either an empty vector or a vector carrying SIRT1. HDAC1 was then immunoprecipitated, and its activity was measured as described in b (*p < 0.01, unpaired t-test). d, Sequence and structural information from an already crystallized HDAC1 ancestor from the hyperthermophilic bacterium Aquifex aeolicus (PMID: 10490031) was used to generate a computational model predicting the tertiary structure of HDAC1. Identical domain color scheme was utilized in domain illustration and predicted structure rendering. Green indicates the position of the lysine residue, K432.
Supplementary Figure 4. a, Hdac1 F/F neurons infected with either eGFP or Cre-eGFP were treated with either vehicle or 5μM etoposide for 1h, following which the cells were lysed and levels of phosphorylated ATM were compared by western blotting. b, Hdac1 F/F neurons infected as in a were treated with either vehicle or 2μM etoposide for 1h, following which the cells were fixed and stained with antibodies to γH2AX. Per-Cell γH2AX intensity was then quantified. c, Mouse primary neurons transfected with the indicated HDAC1 constructs were treated with 2μM etoposide for 1h, and γ H2AX intensity was quantified using per-cell image analysis. Analysis was limited to transfected cells exclusively. Scale bar = 5μm. d, Hdac1 F/F neurons were infected as in a and treated with 5μM etoposide for 30 min. Cells were then lysed either immediately or following recovery after etoposide washout for an additional 30 min. The lysates were then electrophoresed and processed as in Figure 4c. e, Neurons incubated with 10μM compound#10 for 1.2 h were treated with 5μM etoposide for 1h, and fixed and stained with antibodies against γH2AX. Graph indicates quantification of γ H2AX intensity. Scale bar = 10μm. Quantification shown below (** p < 0.001, one-way ANOVA). f, Cultured primary neurons expressing the indicated proteins were incubated with either vehicle or compound#10. The neurons were then treated with etoposide (2μM) for 1h, following which the cells were fixed and stained as in Fig. 5d (* p < 0.05, one-way ANOVA). g, HT22 cells were transfected with the indicated constructs. Cells were then lysed and eGFP-tagged proteins were immunoprecipitated and blotted with antibodies against myc. h, The activity of recombinant HDAC1-flag (100ng) incubated either in the presence or absence of the SIRT1 activator, compound#10 (1.1μM final), was measured using a fluorescence-based HDAC enzymatic activity assay as described in Supplementary Fig. 3b.
Supplementary Figure 5

Supplementary Figure 5. Model. Following DSB formation, DNA ends are recognized by the MRE11 complex and ATM, and DNA end binding by KU70/80 culminates in DSB repair through NHEJ. SIRTI function is crucial in a number of these early events. SIRTI is rapidly recruited to DSBs in an ATM-dependent manner and in turn, stabilizes ATM at DSB sites, and stimulates ATM autophosphorylation and the phosphorylation of ATM targets, including H2AX. In addition, SIRTI also stabilizes NBS1. In this manner, SIRTI facilitates in transduction of the DSB signal (left arrow). SIRTI also facilitates DSB repair through its actions on HDAC1, which is recruited to DSBs in a SIRTI-dependent manner. SIRTI-mediated HDAC1 deacetylation allows HDAC1 to target residues such as H4K16Ac and H3K56Ac, whose deacetylation is essential for DNA repair through NHEJ (right arrow).

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