The regulation of SIRT2 function by cyclin-dependent kinases affects cell motility

Ruwin Pandithage,1 Richard Lilischkis,1 Kai Harting,2 Alexandra Wolf,1 Britta Jedamzik,1 Juliane Lüscher-Firzlaff,1 Jörg Vervoorts,1 Edwin Lasonder,3 Elisabeth Kremmer,4 Bernd Knöll,2 and Bernhard Lüscher1

1Abteilung Biochemie und Molekularbiologie, Institut für Biochemie, Universitätshaus, Rheinische-Westfälische Technische Hochschule Aachen University, 52057 Aachen, Germany
2Interkulturelles Institut für Zeitschriftologie, Abt. Molekularbiologie, Universität Tübingen, 72076 Tübingen, Germany
3Netherlands Centre for Molecular Life Sciences, Centre for Molecular and Biomolecular Informatics, 6500 HB Nijmegen, Netherlands
4Deutsches Forschungszentrum für Gesundheit und Umwelt, Institut für Molekulare Immunologie, 81377 München, Germany

Cytoplasm-dependent kinases (Cdks) fulfill key functions in many cellular processes, including cell cycle progression and cytoskeletal dynamics. A limited number of Cdk substrates have been identified with few demonstrated to be regulated by Cdk-dependent phosphorylation. We identify on protein expression arrays novel cyclin E–Cdk2 substrates, including SIRT2, a member of the Sirtuin family of NAD+-dependent deacetylases that targets α-tubulin. We define Ser-331 as the site phosphorylated by cyclin E–Cdk2, cyclin A–Cdk2, and p35–Cdk5 both in vitro and in cells. Importantly, phosphorylation at Ser-331 inhibits the catalytic activity of SIRT2. Gain- and loss-of-function studies demonstrate that SIRT2 interferes with cell adhesion and cell migration. In postmitotic hippocampal neurons, neurite outgrowth and growth cone collapse are inhibited by SIRT2. The effects provoked by SIRT2, but not those of a nonphosphorylatable mutant, are antagonized by Cdk-dependent phosphorylation. Collectively, our findings identify a posttranslational mechanism that controls SIRT2 function, and they provide evidence for a novel regulatory circuitry involving Cdks, SIRT2, and microtubules.

Introduction

Cdks are heterodimeric enzymes with one catalytic and one regulatory subunit. Dimerization of these two subunits is essential for kinase activity. As the name suggests, some of the regulatory subunits are cyclins, including cyclin E and A, that are synthesized in a cell cycle–dependent manner. These cyclin–Cdk complexes play essential roles in controlling different phases of and progression through the cell cycle (Nurse, 2000; Sherr and Roberts, 2004). However, other regulatory subunits have been identified that are expressed and function independently of the cell cycle (Nebreda, 2006). These include T-type cyclins and cyclin K, which associate with Cdk9 to form distinct positive transcription elongation factor b complexes and cyclin H–Cdk7, which are part of the general transcription factor complex transcription factor II H. These kinases are critical in regulating distinct steps in transcription, including the phosphorylation of components of the mediator complex and the catalytic subunit of the RNA polymerase II complex (Zurita and Merino, 2003; Marshall and Grana, 2006). Furthermore, Cdk5 associates with two regulatory subunits, p35 and 39, and these complexes are expressed primarily in postmitotic neurons as well as in other nonproliferating cells. Cdk5 has been attributed key functions during brain development, including regulation of neuronal survival, cell migration during cortical layering, neurite outgrowth, axon guidance, and synapse function (Dhavan and Tsai, 2001; Nikolic, 2004; Xie et al., 2006).

To obtain further insight into the role of Cdk-dependent regulation of cellular processes, we sought to identify novel substrates for such kinases. We chose cyclin E–Cdk2 because this kinase is an important regulator of the G1 to S-phase transition and is deregulated in a substantial fraction of human tumors (Musgrove, 2006). Indeed, elevated cyclin E expression has been linked to a poor prognosis in human breast cancer (Keyomarsi et al., 2003). Furthermore, the cyclin E–Cdk2 kinase is activated...
in response to several oncoproteins including MYC and the adeno-
viral E1A protein, supporting a role of this kinase in tumori-
genesis (Amati et al., 1998; Luscher, 2001). Among the cyclin E–
Cdk2 substrates are proteins controlling cell cycle progression,
the centrosome cycle, replication, and several transcriptional
regulators (Malumbres and Barbacid, 2005). Cdk2 not only
associates with cyclin E but also with cyclin A, and the two com-
plices share several substrates. In addition, Cdk2 and 5 show
similar substrate specificities (Dhavan and Tsai, 2001). In this
paper we identify 26 cyclin E–Cdk2 substrates, including SIRT2,
a member of the Sirtuin family that consists of seven members,
SIRT1–7, in mammals (Haigis and Guarente, 2006; Michan and
Sinclair, 2007). Sirtuins are class III histone deacetylases (HDAC)
that require NAD⁺ as a cofactor and deacetylate Lys residues.

Sirtuins can be found in different compartments within the cell
regulating a variety of processes, including many aspects of
transcription, the lifespan of organisms, neuroprotection, tumor
suppression, differentiation, and inflammation (Haigis and
Guarente, 2006; Michan and Sinclair, 2007). SIRT2 is the only
Sirtuin family member that is preferentially localized in the cyto-
plasm but, in addition, has also been implicated in nuclear func-
tions (Dryden et al., 2003; North et al., 2003; Vaquero et al.,
2006; Wilson et al., 2006; North and Verdin, 2007a).

Reversible acetylation of proteins at the ε-amino group of
Lys residues has been recognized as an important posttransla-
tional mechanism to control nuclear protein function, including
histones and transcription factors (Kouzarides, 2000). In contrast,
relatively little is known about acetylation/deacetylation of pro-
teins outside the nucleus. Recent evidence, however, suggests
that several cytoplasmic proteins are acetylated (Kim et al., 2006).
Most notably, α-tubulin is acetylated at Lys-40 (K40), a modifi-
cation that has been suggested to enhance microtubule stability
(North et al., 2003). Although the acetyl transferases that mod-
ify K40 are not known, two deacetylases that physically inter-
act, SIRT2 and HDAC6, have been implicated in removing the modifi-
cation (Hubbert et al., 2002; Dryden et al., 2003; North
et al., 2003; Zhang et al., 2003). It has been suggested that
SIRT2 affects progression through mitosis in response to stress
and that SIRT2 is regulated in mitosis by phosphorylation (Dryden
et al., 2003; Inoue et al., 2007; North and Verdin, 2007b).

In this paper, we have identified in SIRT2 a single Cdk2 and 5
phosphorylation site, Ser-331 (S331), C-terminal of the catalytic
domain. Phosphorylation at S331 inhibits the enzymatic activity
of SIRT2. The functional analysis of SIRT2 and phosphorylation
site mutants revealed that this enzyme interferes with cell adhesion
in tumor cells, cell migration in fibroblasts, and neurite outgrowth
and growth cone motility in neurons. Importantly, these SIRT2-
mediated effects are antagonized by Cdk-dependent phosphory-
lation at S331. Collectively, our findings define a posttranslational
mechanism that regulates SIRT2 function both in vitro and in cells.

Results

Identification of novel cyclin-Cdk substrates

To identify novel cyclin E–Cdk2 substrates, high-density protein
arrays on polyvinylidine difluoride filters were phosphorylated
with recombinant baculoviral cyclin E-Cdk2 and γ[32P]ATP
(Fig. 1 A). The clones corresponding to 96 prominent substrates
were sequenced and revealed 42 clones that expressed the ORF
in frame with the N-terminal tag. These represented 26 different
proteins, some known Cdk substrates, with potential phosphory-
site sequences and cyclin binding motifs (Table I). These substrates,
bacterially expressed HIS or GST fusion proteins, were verifi-
ed in vitro kinase assays using different cyclin–Cdk complexes.
Similar amounts of kinase activities were used as assessed with
Rb and histone H1 as substrates (Fig. 1 B). All proteins identi-
fi ed in the screen, except one (RPL14), were phosphorylated by
cyclin E–Cdk2, demonstrating that these are true positive in vitro
substrates (Table I and Fig. S1, available at http://www.jcb.org/
cgi/content/full/jcb.200707126/DC1). As expected, many of the
cyclin E–Cdk2 substrates were also phosphorylated by cyclin A–Cdk2 and, strikingly, by cyclin D3–Cdk4 but not by cyclin B–Cdk1 or cyclin D1–Cdk4 (Table I and Fig. S1).
We observed cyclin D3 phosphorylation of the purified cyclin D3–
Cdk4 complex (Fig. 1, B and C; and Fig. S1), although D-type
cyclins have not been described to be autophosphorylated.
However, because this phosphorylation was completely repressed
by p16INK4A, an inhibitor of D-type kinase complexes, it is most
likely mediated by Cdk4 (Fig. S1 A). In this paper, we chose
to further investigate SIRT2, a member of the Sirtuin family of
NAD⁺-dependent deacetylases.

Phosphorylation of SIRT2 by Cdk5

The findings from the screen were verifi ed by phosphorylating a
GST-SIRT2 fusion protein by the different purifi ed kinase com-
plexes (Fig. 1 and Fig. S2, available at http://www.jcb.org/cgi/
content/full/jcb.200707126/DC1). SIRT2 was a substrate for
cyclin E–Cdk2 and, to a lesser extent, cyclin A–Cdk2 (Fig. 1 C).
In addition SIRT2 was weakly phosphorylated by cyclin D3–
Cdk4 and cyclin B–Cdk1 but not by cyclin D1–Cdk4 (Fig. 1 C).
Inspection of the human SIRT2 protein sequence revealed a sin-
gle consensus sequence for Cdk5, S331/PPK, which is C terminal
of the catalytic domain (Fig. 1 D) and is conserved in mouse
SIRT2. Similar phosphorylation sites at comparable positions
relative to the catalytic domains are found in the SIRT family
members SIRT1, 6, and 7 (Fig. 1 D) and are phosphorylated by
Cdk2 in vitro (not depicted). To address whether S331 is phos-
phorylated by Cdk2, GST-SIRT2 fusion proteins with S331 muta-
ted to Ala, Asp, or Glu were phosphorylated with recombinant
cyclin E–Cdk2 or cyclin A–Cdk2. Mutation of S331 abolished
Cdk2-dependent phosphorylation (Fig. 1 E; and Fig. S2, A–C).
The reactions were specifi c because roscovitine, a Cdk inhibi-
tor, abolished SIRT2 phosphorylation (Fig. S2, A and C). In ad-
dition, S331 phosphorylation by cyclin E–Cdk2 in vitro was
confi rmed by mass spectrometry analysis (Fig. S2 D).

SIRT2 is highly expressed in the nervous system (Fig. S3,
available at http://www.jcb.org/cgi/content/full/jcb.200707126/
DC1), which is in agreement with previous reports (Li et al.,
2007; Southwood et al., 2007). Similarly, in the nervous system,
Cdk5 is strongly expressed in postmitotic neuronal cells, unlike
Cdk2, which is down-regulated when neurons exit the cell cycle
and differentiate (Freeman et al., 1994). Therefore, we tested
whether a p35–Cdk5 complex could phosphorylate SIRT2.
**Figure 1. Identification of SIRT2 as a Cdk substrate.** (A) High-density protein arrays (two filters with 37,830 clones preselected for high protein expression by virtue of N-terminal 6xHis tags [Bussow et al., 1998]) were incubated with recombinant human cyclin E–Cdk2 and [γ-32P]ATP, washed, and exposed to x-ray film. Potential substrates appear as double spots, as indicated by circles. A portion of one filter is displayed. (B) cyclin–Cdk complexes were incubated with or without their respective substrates (Cdk4 complexes with GST-pRb 773–928 and Cdk2 and 1 complexes with histone H1) and [γ-32P]ATP. Proteins were resolved by 7–17% SDS-PAGE and visualized by autoradiography (32P, top) or Coomassie blue staining (CB, bottom). Kinase complexes are abbreviated (e.g., D1–K4 for cyclin D1–Cdk4). (C) Bacterially expressed GST-SIRT2 full-length fusion protein was phosphorylated with the indicated kinases as described in B. (D) Schematic comparison of Saccharomyces cerevisiae Sir2 with human SIRT1, 2, 6, and 7. The catalytic domains and the potential Cdk phosphorylation sites are indicated. (E) Bacterially expressed GST-SIRT2 fusion proteins, as indicated, were phosphorylated with 25 fM cyclin E–Cdk2. GST and histone H1 served as controls. Protein analysis was performed as in B. (F) The experiment was performed as in E with 3 fM of recombinant p35–Cdk5. For a control, 25 μM roscovitine was added to inhibit Cdk5 activity.
### Table I. In vitro phosphorylation of potential cyclin E–Cdk2 substrates by different cyclin–Cdk complexes

<table>
<thead>
<tr>
<th>Gene</th>
<th>ORF of RZPD clone</th>
<th>Name/biological process</th>
<th>Potential cyclin E–Cdk2 substrate</th>
<th>Phosphorylation sites and cyclin binding motifs in the RZPD clone</th>
<th>Phosphorylation of the RZPD clone by kinases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUMA1**</td>
<td>245 aa C-term</td>
<td>Nuclear mitotic apparatus protein 1/spindle apparatus organization</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>LOC201191</td>
<td>231 aa C-term</td>
<td>Hypothetical protein</td>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PIP5K1C</td>
<td>200 aa C-term</td>
<td>Phosphatidylinositol-4-phosphate 5 kinase, type 1/3/phosphatidylinositol signaling</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CCNL2</td>
<td>136 aa C-term</td>
<td>cyclin L2/RNA processing</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>RASL11B</td>
<td>118 aa C-term</td>
<td>RAS-like, family 11, member B/signaling?</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>SIRT2</td>
<td>full-length</td>
<td>Sirtuin [silent mating type information regulation 1 homologue] 2 (S. cerevisiae) / NAD+-dependent histone/protein deacetylase</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FU13111</td>
<td>full-length</td>
<td>Hypothetical protein FU13111/unknown</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>SUPT5H*</td>
<td>120 aa C-term</td>
<td>Suppressor of Ty 5 homologue</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>STMN2*</td>
<td>full-length</td>
<td>Stathmin-like 2/microtubule destabilization in neuronal growth</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LOC146909</td>
<td>114 aa C-term</td>
<td>Hypothetical protein LOC146909/unknown</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>EEF1G</td>
<td>211 aa C-term</td>
<td>Eukaryotic translation elongation factor 1/translation</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TRIT1</td>
<td>99 aa C-term</td>
<td>tRNA isopentenyltransferase 1/RNA modification</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LOC388799</td>
<td>89 aa C-term</td>
<td>Hypothetical protein LOC388799/unknown</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>STUB1</td>
<td>full-length</td>
<td>STIP1 homology and U-box-containing protein 1/protein turnover</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>SRRM2</td>
<td>98 aa C-term</td>
<td>Serine/arginine repetitive matrix 2/RNA processing</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TALDO1</td>
<td>full-length</td>
<td>Transaldolase 1/metabolism</td>
<td>3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>SFRS1</td>
<td>243 aa C-term</td>
<td>Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)/RNA processing</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SNAPAP</td>
<td>full-length</td>
<td>SNAP-associated protein/exocytosis of synaptic vesicles</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LOC339287</td>
<td>90 aa C-term</td>
<td>Hypothetical protein LOC339287/unknown</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>FU12949</td>
<td>248 aa C-term</td>
<td>Hypothetical protein FU12949/unknown</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>MAP2</td>
<td>376 aa C-term</td>
<td>MAP2/microtubule assembly in neurogenesis</td>
<td>16</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>RPL14</td>
<td>211 aa C-term</td>
<td>Ribosomal protein L14/translation</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FNBP3</td>
<td>245 aa C-term</td>
<td>Formin binding protein 3/splicing?</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PRC1*</td>
<td>full-length</td>
<td>Protein regulating cytokinesis 1/cytokinesis</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>FU13305</td>
<td>162 aa C-term</td>
<td>Hypothetical protein FU13305/unknown</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>ING5</td>
<td>full-length</td>
<td>Inhibitor of growth family, member 5/p53 pathway, replication</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*The kinases used are abbreviated (e.g., D1–K4 for cyclin D1–Cdk4). The substrates are phosphorylated strongly (+), weakly (±), or not at all (−) by the respective kinase.

*Previously reported Cdk substrate (Stachora et al., 1997; Gavet et al., 1998; Sun and Schatten, 2006).

*For ING5 and SIRT2, GST-tagged full-length proteins were used in the kinase assays.

*Previously reported cyclin E–Cdk2 substrate (Jiang et al., 1998).

---

**Table Note:**
- *NUMA1:* Nuclear mitotic apparatus protein 1/spindle apparatus organization
- *LOC201191:* Hypothetical protein
- *PIP5K1C:* Phosphatidylinositol-4-phosphate 5 kinase, type 1/3/phosphatidylinositol signaling
- *CCNL2:* Cyclin L2/RNA processing
- *SUPT5H:* Suppressor of Ty 5 homologue
- *STMN2:* Stathmin-like 2/microtubule destabilization in neuronal growth
- *LOC146909:* Hypothetical protein LOC146909/unknown
- *EEF1G:* Eukaryotic translation elongation factor 1/translation
- *TRIT1:* RNA isopentenyltransferase 1/RNA modification
- *LOC388799:* Hypothetical protein LOC388799/unknown
- *STUB1:* STIP1 homology and U-box-containing protein 1/protein turnover
- *SRRM2:* Serine/arginine repetitive matrix 2/RNA processing
- *TALDO1:* Transaldolase 1/metabolism
- *SFRS1:* Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)/RNA processing
- *SNAPAP:* SNAP-associated protein/exocytosis of synaptic vesicles
- *LOC339287:* Hypothetical protein LOC339287/unknown
- *FU12949:* Hypothetical protein FU12949/unknown
- *MAP2:* MAP2/microtubule assembly in neurogenesis
- *RPL14:* Ribosomal protein L14/translation
- *FNBP3:* Formin binding protein 3/splicing?
- *PRC1:* Protein regulating cytokinesis 1/cytokinesis
- *FU13305:* Hypothetical protein FU13305/unknown
- *ING5:* Inhibitor of growth family, member 5/p53 pathway, replication
Similar to cyclin-E–Cdk2 and cyclin-A–Cdk2 complexes, p35–Cdk5 was capable of phosphorylating SIRT2 at S331 (Fig. 1 F), suggesting that this site can be phosphorylated both in cycling and differentiated cells.

To corroborate the in vitro kinase assays, we analyzed the phosphorylation of SIRT2 in cells (Fig. 2). HA-tagged SIRT2 or SIRT2-S331A was expressed in HeLa cells and labeled with [32P]orthophosphate. Although SIRT2 was phosphorylated, the S331A mutant was only poorly labeled (Fig. 2 A). Moreover, coexpression of the Cdk2 inhibitor p27KIP1 abolished SIRT2 phosphorylation (Fig. 2 A). We also generated HEK293 cells stably expressing a tagged version of SIRT2 (N–tandem affinity purification [TAP]–SIRT2; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200707126/DC1). The analysis of TAP-SIRT2 isolated from exponentially growing cells by mass spectrometry confirmed SIRT2 phosphorylation at S331 (Fig. 2 B).

In support of this, a peptide that contains S331 was identified as phosphopeptide in a global screen (Olsen et al., 2006). In addition, SIRT2 phosphorylation was high in cells blocked in S-phase and in prometaphase by hydroxyurea and nocodazole, respectively, but low in cells blocked in metaphase by colcemide when analyzed by [32P]orthophosphate labeling (Fig. 2 C) and mass spectrometry (not depicted). This correlates with active cyclin E–Cdk2 and cyclin A–Cdk2 kinase complexes, implicating these kinases in SIRT2 phosphorylation. In summary, these findings define S331 as the major cell cycle–regulated Cdk-dependent phosphorylation site of SIRT2.

To expand on the observations described in the previous paragraph, we addressed whether endogenous SIRT2 is phosphorylated at Ser-331. We generated mAbs against a human SIRT2 Ser-331 phosphorylated peptide. The antibodies were screened on HA-SIRT2 and HA-SIRT2-S331A. Although mAb 7G5 recognized both proteins, mAb 6B5P detected only HA-SIRT2 (Fig. S2 E). In support of this, the epitope was lost when overexpressed HA-SIRT2 was phosphatase treated, indicating that mAb 6B5P is phosphospecific (Fig. S2 F). Importantly the 6B5p epitope was generated when bacterially expressed GST-SIRT2 was phosphorylated by cyclin E–Cdk2 (Fig. 2 D).

We then addressed whether endogenous SIRT2 was phosphorylated at Ser-331. Because in multiple cell lines analyzed SIRT2 levels were low and did not allow direct analysis by Western blotting, we examined Ser-331 phosphorylation upon immunoprecipitation of endogenous SIRT2. HEK293 cells were arrested in S-phase using hydroxyurea to enhance cyclin E–Cdk2–dependent phosphorylation and treated the cells for the last 2 h with roscovitine, a Cdk2 inhibitor. In HEK293 cells, the two described SIRT2 isoforms, a consequence of alternative splicing (North and Verdin, 2007b), were detected with the phosphospecific mAb 6B5P (Fig. 2 E). Roscovitine treatment reduced SIRT2 phosphorylation by at least 60% and the 6B5P epitope was sensitive to phosphatase treatment (Fig. 2 E). Reduced phosphorylation correlated with the appearance of SIRT2 protein species with slightly increased mobility (Fig. 2 E, double bands). To address cell cycle regulation, serum-starved primary human diploid fibroblasts (HDFs) were stimulated with serum and entered S-phase by 18 h as monitored by BrdU incorporation (unpublished data). Although overall levels of SIRT2 (only the larger isoform was detectable in HDFs) did not change, phosphorylation increased (Fig. 2 F). This observation is in agreement with cyclin-E–Cdk2 functioning as a SIRT2 kinase (Fig. 1 E), the sensitivity of SIRT2 phosphorylation to the cyclin E–Cdk2 inhibitor p27 (Fig. 2 A), the cell cycle analysis (Fig. 2 C), and the sensitivity of SIRT2 phosphorylation to roscovitine (Fig. 2 E).

To further strengthen the functional interaction of SIRT2 with Cdk complexes, we analyzed whether these proteins interacted physically. Coimmunoprecipitation experiments revealed that SIRT2 and cyclin E–Cdk2 interacted when coexpressed in HEK293 cells (Fig. 2 G) and cyclin E bound to SIRT2 in vitro pulldown experiments (not depicted). Furthermore, TAP-SIRT2, TAP-SIRT2-S331A, and TAP-SIRT2-H150Y, but not TAP-SIRT2-S331D, interacted with endogenous cyclin E in HEK293 cells stably expressing the SIRT2 proteins (Fig. S2 G). Similarly, p35 and Cdk5 were coimmunoprecipitated with SIRT2 upon overexpression in HEK293 cells (Fig. 2 H). Interaction of endogenous p35–Cdk5 and SIRT2 was demonstrated by coimmunoprecipitation from P14 mouse brain extracts (hippocampus and cortex; Fig. 2, I and J). Furthermore, in addition to previous reports showing SIRT2 expression mainly in oligodendroglial cells (Li et al., 2007; Southwood et al., 2007), primary hippocampal neurons expressed SIRT2 throughout the cell body in the neurites and growth cones (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200707126/DC1). Moreover, SIRT2 colocalized with p35, the regulatory subunit of Cdk5 (Fig. S5, A–D), and SIRT2 expression overlapped with acetylated α-tubulin in neuronal growth cones (Fig. S5, E–G). Thus p35, which has recently been shown to directly bind to microtubules (Hou et al., 2007), colocalizes with SIRT2 and α-tubulin. In summary, these findings support our phosphorylation analysis and the notion that Cdk complexes, SIRT2, and α-tubulin interact functionally.

Phosphorylation at S331 inhibits the catalytic activity of SIRT2

Next we assessed the functional relevance of S331 phosphorylation. No difference in the subcellular distribution of SIRT2 and SIRT2-S331A mutants or the stability of these proteins could be observed (unpublished data). Importantly, however, S331 phosphorylation affected the catalytic activity of SIRT2. GST-SIRT2 showed robust NAD+-dependent deacetylase activity toward core histones, which was blocked by nicotinamide (Fig. 3 A). GST-SIRT2-S331A had comparable deacetylase activity when adjusted to protein concentrations (Fig. 3, A and B). In contrast, GST-SIRT2-S331D, a mutation that may mimic phosphorylation at S331, showed reduced activity (Fig. 3, A and B). All mutants with the active center His-150 (H150) changed to Tyr were catalytically inactive as reported previously (Frye, 1999). These findings suggested that phosphorylation at S331 inhibits the enzymatic activity of SIRT2. Indeed, when GST-SIRT2 was incubated in the presence of cyclin E–Cdk2 or cyclin A–Cdk2, the catalytic activity of SIRT2 was repressed (Fig. 3, C and D). These kinase-dependent effects required S331 because GST-SIRT2-S331A was not inhibited. Moreover, roscovitine reversed the repressive effect (Fig. 3 C). It is noteworthy that although p35–Cdk5 phosphorylated S331 (Fig. 1 F), the
Figure 2. SIRT2 is phosphorylated at serine 331 in cells and interacts with Cdk complexes. (A) HEK293 cells were transiently transfected with plasmids encoding 10 μg HA-SIRT2, 10 μg HA-SIRT2-S331A, and 5 μg p27, as indicated, and metabolically labeled with [32P]orthophosphate. SIRT2 was then immunoprecipitated via its HA-tag. The top shows an autoradiography, the bottom shows Western blots for HA-SIRT2 (mAb 3F10) and p27 KIP1 (C-19). (B) N-TAP-SIRT2 was purified from HEK293 cells. The fragmentation spectrum of parent ion m/z 1144.5163, 2 + (mass accuracy, 2.8 ppm) is shown. Mascot searches against the Uniprot database identified that this peptide unambiguously phosphorylated at position Ser-331 with a Mascot ion score of 68 (Expectance value, 1.9*E-4). The phosphopeptide was sequenced eight times from two unique overlapping peptide sequences because of the presence of miscleaved tryptic sites. The y-ion fragmentation ladder starting from the C terminus is shown in red. The phosphorylation site was mapped by the detection of the y1 fragment ion in the linear ion trap at m/z 411.3, which corresponds to the sequence pSPK. (C) HEK293 cells were transiently transfected with a plasmid encoding 10 μg HA-SIRT2 and treated with 200 μM hydroxyurea, 400 ng/ml nocodazole, or 200 ng/ml colcemide for 20 h and then metabolically labeled with [32P]orthophosphate. The SIRT2 analysis was performed as described in A. White lines indicate that intervening lanes have been spliced out. (D) Bacterially expressed and purified GST-SIRT2 or GST was phosphorylated by recombinant cyclin E–Cdk2 in the presence or absence of ATP as indicated. Half of the reactions were analyzed by Coomassie blue [CB] staining, the other half were subjected to Western blot analysis using the mAb 6B5 that is specific for Ser-331 phosphorylated SIRT2. (E) HEK293 cells were treated as indicated with hydroxyurea (HU) for 16 h and with roscovitine (Rosc) for the last 2 h. SIRT2 was immunoprecipitated using the polyclonal serum 748 from RIPA lysates of 6 × 10⁶ cells. The immunoprecipitated proteins were phosphatase or mock treated and the proteins were analyzed by Western blotting with the indicated mAbs. (F) Primary HDFs were serum starved and then treated with 10% serum for the indicated times. Parallel samples were analyzed for BrdU incorporation, indicating that the cells started to enter S-phase 18 h after serum addition. SIRT2 was immunoprecipitated from lysates of roughly 4 × 10⁶ cells per sample and detected with the indicated mAbs.
poor catalytic activity and the instability of this commercially available complex did not allow us to directly test its in vitro effects on SIRT2 catalytic activity. However, because p35–Cdk5 also phosphorylates S331, it is highly likely that the deacetylase activity of SIRT2 would be inhibited.

Finally, we addressed whether SIRT2 expressed in mammalian cells was catalytically active. HA-SIRT2 and mutants were expressed in HEK293 cells, immunoprecipitated, and assayed for HDAC activity (Fig. 3, E and F). As for the bacterially expressed proteins, SIRT2 had comparable activities to SIRT2-S331A, whereas the phospho-mimicking mutants were less active when compared with input. In addition, nicotinamide inhibited the catalytic activity and SIRT2-H150Y was inactive (Fig. 3, E and F). Furthermore, SIRT2 and SIRT2-S331A, but not SIRT2-H150Y, deacetylated α-tubulin in brain extracts in a NAD⁺-dependent manner (Fig. 3 G). Finally, we coexpressed SIRT2 with cyclin E–Cdk2 or p35–Cdk5 and measured HDAC activity of immunoprecipitated SIRT2. Both kinases inhibited, whereas p27Kip1 slightly stimulated, the catalytic activity of SIRT2 (Fig. 3 H). Collectively, our data demonstrate that phosphorylation at S331 represses the enzymatic activity of SIRT2.

SIRT2 regulates cell adhesion and cell migration

It had been suggested that SIRT2 affects passage through mitosis, possibly as a consequence of altered α-tubulin acetylation (Dryden et al., 2003; North et al., 2003). Therefore, we tested whether SIRT2 and mutants altered the cell cycle in HeLa and HEK293 cells. No systematic effects on cell cycle distribution and proliferation could be measured (Fig. S4). However, we
noticed cell detachment upon induction of SIRT2-S331A in HEK293 cells and analyzed this further. SIRT2 and SIRT2-S331A resulted in a 2.5- and 4-fold increase, respectively, of detached cells (Fig. 4, A and B). Importantly, the catalytically inactive mutant SIRT2-H150Y and the phospho-mimicking mutant SIRT2-S331D with reduced catalytic activity had no effect (Fig. 4 B). SIRT2-mediated inhibition of cell adhesion coincided with a reduced α-tubulin acetylation (Fig. 4 C). Conversely, knockdown (KD) of SIRT2 using a siRNA that targets both human and mouse SIRT2 increased substratum adhesion of transfected cells and resulted in an increase of α-tubulin acetylation (Fig. 4, D and E). Cyclin E–Cdk2 or p35–Cdk5 efficiently inhibited SIRT2-induced cell detachment (Fig. 4 D) but had no effect on SIRT2-S331A (Fig. 4 F). To test whether SIRT2 also affected cell adhesion of primary cells, we expressed SIRT2 and mutants in mouse embryonic fibroblasts (MEFs). Similar to the observation in HEK293 cells, SIRT2 and SIRT2-S331A inhibited attachment of MEF cells to laminin-coated coverslips in comparison with the catalytically inactive SIRT2-H150Y (Fig. 4 G).

In addition to cell adhesion, SIRT2 function was explored in migration of MEF cells (Fig. 4 H). A mechanical scratch was applied to MEF monolayers transiently expressing SIRT2 or SIRT2 mutants and re-invasion of the cleared area by cells was monitored. In line with the results obtained in cell adhesion (Fig. 4, A–F), SIRT2 slightly, and SIRT2-S331A significantly, blocked migration of MEFs (Fig. 4 H). These findings suggest that altering the SIRT2 activity in cells affects the interaction of cells with the substratum, possibly by altering the stability of microtubules because of differential acetylation.

**SIRT2 overexpression inhibits neurite outgrowth**

The intimate link between p35–Cdk5 and SIRT2, as shown by the ability of Cdk5 to phosphorylate SIRT2 (Fig. 1 F), which is shown by co-immunoprecipitation studies (Fig. 2, I and J) and deacetylation assays (Fig. 3 H), suggests that SIRT2 functions as a novel downstream effector of Cdk5. Microtubules possess pivotal functions in regulating multiple aspects of neuronal motility, including migration, neurite outgrowth, and growth cone turning (Dent and Gertler, 2003; Gordon-Weeks, 2004). Cdk5 has been implicated in all these processes (Dhavan and Tsai, 2001; Nikolic, 2004). Therefore, we first assessed the role of SIRT2 in neurite formation and protrusion (Fig. 5). Primary mouse hippocampal neurons were co-electroporated with SIRT2 and GFP-expressing plasmids and cultured for 2 d on laminin to promote neurite outgrowth. The cells were then stained for acetylated α-tubulin and the neurite length of GFP-positive cells was determined (Fig. 5). SIRT2 and, more profoundly, SIRT2-S331A reduced neurite length, whereas SIRT2-H150Y, SIRT2-H150Y-S331A, SIRT2-S331E, and SIRT2-S331D had no effect (Fig. 5, A and B; and not depicted). Of note, quantification of the neurite length (Fig. 5 B) underestimated the consequence of SIRT2-S331A expression because in more than half of the cells, neurite formation was completely abolished, resulting in a rounded-up phenotype (Fig. 5, A and C). This was not observed for SIRT2, which can be phosphorylated and thereby inhibited. Acetylation of α-tubulin was reduced by SIRT2-S331A and, to a lesser extent, by SIRT2 (Fig. 5 D; and not depicted). In agreement with SIRT2 being a novel Cdk5 effector, SIRT2-mediated, but not SIRT2-S331A-mediated, neurite outgrowth inhibition was rescued at least partially by p35–Cdk5 (Fig. 5 E).

The overexpression findings were complemented by KD of SIRT2, which resulted in a significant increase of neurite length (Fig. 5, F and G). Notably, the number of neurons with very long neurites (>150 μm) was increased almost threefold in SIRT2 KD cells (Fig. 5 G). Finally, we also observed an increase in total neurite number (on average 25%) per neuron upon SIRT2 KD (unpublished data), further stressing SIRT2’s inhibitory role in neurite outgrowth. To address potential off-target effects, we used a second mouse-specific SIRT2 siRNA that also increased neurite length (unpublished data), whereas a human-specific SIRT2 siRNA was indistinguishable from siLUC. In summary, these findings demonstrate a decisive role of SIRT2 in neurite outgrowth of primary neurons, which is in agreement with its role in oligodendroglial arborization (Li et al., 2007).

**SIRT2 impairs cytoskeletal growth cone dynamics stimulated by repulsive axon guidance cues**

A well-established paradigm to study axon guidance in tissue culture is the so-called growth cone collapse assay. Members of the ephrin-A family trigger growth cone repulsion through stimulating EphA receptor tyrosine kinases, thereby preventing growth cones from turning into aberrant target areas of the brain (Knoll and Drescher, 2002; Pasquale, 2005). Cdk5 is a key mediator of EphA receptor-mediated repulsion (Cheng et al., 2003; Fu et al., 2007). Consequently, we explored the role of SIRT2 in growth cone collapse. Hippocampal neurons revealed typical well-elaborated growth cones with finger-like filopodial and veil-like lamellipodial structures in response to Fc control proteins (Fig. 6 A). In contrast, ephrin-A5–Fc induced a complete breakdown of the growth cone F-actin cytoskeleton within 30 min, leaving the neurites with a retracted neurite shaft (Fig. 6, A and B; Knoll et al., 2006). Importantly the growth cones of neurons expressing SIRT2 or SIRT2-S331A, but not SIRT2-S331 A-H150Y, SIRT2-S331E, or SIRT2-S331D, were resistant to ephrin-A5–Fc–induced collapse (Fig. 6, A–C; and not depicted). Thus, ephrin-A5–Fc collapsed 60–70% of the growth cones, an effect that was reduced to 30–40% by SIRT2 and SIRT2-S331A (Fig. 6 B). In summary, the reduction in growth cone collapse by SIRT2 and SIRT2-S331A was ~50 and 75%, respectively (Fig. 6 C). Similar to the reduced α-tubulin acetylation in the entire neuron (Fig. 5 D), we determined a reduction of acetylated α-tubulin as compared with the control by 40 and 25% in response to SIRT2-S331A and SIRT2, respectively, in growth cones (not depicted).

F-actin staining was reduced 2.5-fold upon ephrin-A5–Fc treatment (Fig. 6 D). This could not be further enhanced by co-expressing p35–Cdk5, although the expression of this kinase complex slightly reduced F-actin staining in the absence of ephrin-A5–Fc. Importantly, and in accordance with the morphological assessment of growth cone collapse (Fig. 6, A–C), the expression of SIRT2 and SIRT2-S331A blocked the ephrin-A5–Fc–induced growth cone collapse as revealed by F-actin staining.
ephrin-A5-Fc a strong effect on growth cones was observed (Fig. 6 E). Growth cones of siSIRT2-treated neurons protruded significantly less filopodia in comparison to siLUC control-treated cells. Thus, in cells in which SIRT2 was knocked down, a collapsed morphology was observed already in the absence of ephrin-A5-Fc. Importantly, the effect of SIRT2, but not of SIRT2-S331A, was reverted by p35-Cdk5 (Fig. 6 D). Because SIRT2 and SIRT2-S331A stabilized growth cones upon ephrin-A5 treatment, we investigated the effect of SIRT2 KD on growth cones, expecting that reduced SIRT2 expression would sensitize these structures to ephrin-A5-Fc treatment. However, already in the absence of ephrin-A5-Fc a strong effect on growth cones was observed (Fig. 6 E). Growth cones of siSIRT2-treated neurons protruded significantly less filopodia in comparison to siLUC control-treated cells. Thus, in cells in which SIRT2 was knocked down, a collapsed morphology was observed already in the absence of ephrin-A5-Fc. In support of this, the KD of SIRT2 also resulted in a collapsed morphology.
Cdks phosphorylate Ser-331 within a Cdk consensus sequence of SIRT2 that is conserved in several Sirtuin family members (Figs. 1, 2, and S2). Mechanistically, phosphorylation at this site represses the enzymatic activity of SIRT2 (Fig. 3), representing the first account of a posttranslational modification linked to SIRT2 enzymatic activity. Recently, the comparable site in an alternatively spliced version of SIRT2 was identified as cyclin B–Cdk1 substrate but no function was assigned to this modification (North and Verdin, 2007b). This may be because of the relative low activity of cyclin B–Cdk1 toward SIRT2 in comparison to the Cdk2 complexes as observed both in vitro and in cells (Figs. 1 and 2).

To date, two SIRT2 substrates, α-tubulin and histone H4, have been described (Dryden et al., 2003; North et al., 2003; North et al., 2007).

in an ~25% reduction in F-actin content in growth cones in the absence of ephrin-A5 (n = 100 growth cones each; unpublished data), which is consistent with morphological alterations observed in these growth cones (Fig. 6 E). In summary, these findings suggest strongly that the EphA-stimulated growth cone collapse is mediated at least in part by the phosphorylation and thereby repression of the deacetylase activity of SIRT2.

Discussion

Sirtuins have received considerable attention because of their role in aging, neuroprotection, and gene transcription (Haigis and Guarente, 2006; Michan and Sinclair, 2007). We identified SIRT2 in a phosphorylation screen with recombinant cyclin E–Cdk2.
Vaquero et al., 2006). SIRT2 deacetylates histone H4 at Lys-16 during G2/M, during which a subfraction of SIRT2 associates with chromatin. Deacetylation of H4K16 was suggested to be relevant for efficient chromatin condensation (Vaquero et al., 2006). The lack of SIRT2 results in a decrease in S-phase and increase in G1-phase in MEFs without apparent effect on mitosis. In contrast, in human tumor cells overexpression of SIRT2 delayed exit from mitosis, probably because of altered α-tubulin acetylation (Dryden et al., 2003). Recently, SIRT2 was shown to inhibit mitotic slippage in cells treated with mitotic spindle drugs (Inoue et al., 2007; North and Verdin, 2007b), suggesting a role in genetic stability. In agreement with this, SIRT2 is down-regulated in certain tumors (Hiratsuka et al., 2003; Matsushita et al., 2005; Voelter-Mahlknecht et al., 2005; Inoue et al., 2007). Our own analysis did not reveal any reproducible effects on mitosis in response to SIRT2, SIRT2 mutants, or KD of SIRT2 in tumor cell lines in the absence of stress (Fig. S4).

We provide evidence that SIRT2 impairs cell motility, at least in part by influencing microtubule dynamics, which is a function regulated by Cdk phosphorylation. α-Tubulin acetylation is associated with stable microtubules (Dent and Gertler, 2003; Westermann and Weber, 2003). We used multiple assays to investigate a role for SIRT2 in cell motility, including adhesion, migration, neurite outgrowth, and growth cone collapse (Figs. 4–6). All the aforementioned processes share signaling through focal contacts leading to attachment/detachment of cells from their substratum. Because microtubules and focal adhesions show extensive crosstalk (Ezratty et al., 2005), it is possible that α-tubulin deacetylation also affects the activities of focal adhesions. An increase in microtubule dynamics because of a reduction in α-tubulin acetylation might modulate focal adhesion turnover and thereby control cell motility. In neurons, an important cross talk between dynamic microtubules and actin filaments takes place at the central/peripheral growth cone interface to regulate target-directed advancement of the growth cone (Dent and Gertler, 2003). Microtubules have been shown to be crucially involved in both neurite outgrowth and chemotactic sensing of guidance cues in the surrounding of navigating growth cones (Gordon-Weeks, 2004). In neurons, acetylated microtubules are polarized toward the starting point.
of the first nerve fiber protrusion (de Anda et al., 2005). It is possible that deacetylation of α-tubulin might block directed protrusion of a neurite, which would be consistent with our observation that SIRT2 inhibits neurite outgrowth (Fig. 5). The most dramatic effects were observed with nonphosphorylatable SIRT2-S331A.

Cdk5 is essential for neurite outgrowth during neuronal differentiation (Nikolic et al., 1996) and is required, together with its regulatory subunits, for the cytoarchitecture of the central nervous system (Dhavan and Tsai, 2001). Cdk5 has been implicated in the regulation of the actin and microtubule network by phosphorylating several proteins associated with these cytoskeletal components, including p27KIP1, PAK1, FAK, doublecortin, and microtubule-associated proteins (MAPs) like MAP1B and tau (Nikolic et al., 1998; Xie et al., 2003; Drewes, 2004; Tanaka et al., 2004; Kawachi et al., 2006). Of interest is the analysis of p27KIP1, which is a substrate of both Cdk2 and 5. However, different sites are phosphorylated. Cdk2 modifies Thr-187, stimulating degradation, and Cdk5 phosphorylates Ser-10, enhancing stability in a cell type–specific manner. The latter is observed in neurons and is necessary for neuronal migration (Kawachi et al., 2006). In other situations, the precise function of Cdk5-dependent phosphorylation may be less well understood; however, in general, stabilization of microtubules is observed. It is noteworthy that Cdk5 was previously shown to be an important downstream effector of the EphA receptor (Cheng et al., 2003; Fu et al., 2007). Hence, the EphA-Cdk5-SIRT2 signaling cascade might ensure full growth cone collapse. In this respect, Cdk phosphorylation and thereby inactivation of SIRT2-mediated α-tubulin deacetylation might contribute toward maintaining stable microtubules. Besides α-tubulin, we cannot rule out additional SIRT2 substrates that might contribute to the cell motility phenotypes reported here. Indeed, the recent identification of several cytoplasmic proteins, which are acetylated (Kim et al., 2006), suggests that SIRT2 and other cytoplasmic deacetylases will have additional substrates that will be important to define in the future.

Cyclin E–Cdk2 is implicated in the development of human tumors (Mugrove, 2006). The identification of SIRT2 as a novel substrate and its role in cell motility suggests that cyclin E–Cdk2 can regulate distinct aspects of the cytoskeleton. This raises the question of how these kinases get access to cytoskeletal components because both cyclin E–Cdk2 and cyclin A–Cdk2 are predominantly nuclear. A recent study demonstrates that both kinase complexes shuttle between the nuclear and cytoplasmic compartments, enabling access to cytoplasmic substrates (Jackman et al., 2002). In addition to its interaction with SIRT2 (Fig. 2), a role of Cdk2 in microtubule dynamics is supported by the identification of stathmin-like 2 (STMN2 or SCG10; Grenningloh et al., 2004; Morii et al., 2006) and MAP2 (Dehmelt and Halpain, 2004) as potential substrates (Table I). STMN2 and MAP2 influence microtubule stability, are regulated by Cdk5, and affect neurite outgrowth (Dent and Gertler, 2003). Collectively, these findings suggest that cyclin E–Cdk2, activated by proliferation signals or oncogenic mutations, regulates microtubule dynamics by phosphorylating several substrates associated with microtubules (Fig. 6 F). This could be relevant for different aspects of tumor cell biology, including elevated cell migration and cell cycle progression.

Materials and methods

Cells, transfections, and assays

HEK293, MEF, and Hela cells were cultured in DME supplemented with penicillin/streptomycin and 10% FCS. Transient transfection assays were performed as described previously (Luscher-Firzlaff et al., 2006). HEK293 Flip-In TANGO 293 cells (Invitrogen) were stably transfected with pDON/FR/TO/N-TAP-SIRT2 or SIRT2-S331A by coexpressing the Flip recombinase. Cell lysates of HDFS were obtained from J. Baron and U. Linzen (Rheinisch-Westfälische Technische Hochschule Aachen University, Aachen, Germany). Preparation and electroporation of primary hippocampal cultures were performed as described previously (Knoll et al., 2006). In brief, hippocampi of P1-P3 mice were incubated in trypsin-ENDA at 37°C for 10 min, followed by washing in HBSS and resuspending in prewarmed DME/10% horse serum (HS). The tissue was triturated using flame-polished Pasteur pipettes and spun down for 5 min at 600 rpm and the pellet was reconstituted in DME/HS. After counting, the cells were pelleted and then resuspended in mouse neuron Nuclear vector solution (Amaxa) as recommended by the manufacturer. A total of 3 μg DNA (2.25 μg of the desired construct and 0.75 μg of permuted EGFP was used) was electroporated per sample. Cultures were plated in neurobasal medium supplemented with B27 supplement (Invitrogen) for 2 d in vitro. The significance of experimental results was determined by t test (two sided), with one asterisk indicating P < 0.05, two asterisks P < 0.01, and three asterisks P < 0.001.

To determine the detached cells, transiently transfected HEK293 cells were identified by analyzing EGFP-positive cells. 2 d after transfection, the number of transacted detached and transfected adherent cells was counted, and from this the rate of detachment was determined. For cell migration, 10^6 MEFs were plated in one well of a 24-well plate, followed by transfection with Lipofectamine with 0.6 μg SIRT2 construct plus 0.2 μg GFP/well. The next day, a scratch was applied and cell migration into the cleared area was monitored 12 and 24 h afterward. The number of GFP-positive cells in and outside the scratch was determined along with bright field recording.

To determine the effect of SIRT2 on α-tubulin acetylation in cells, HEK293 cells were cotransfected with plasmids expressing SIRT2 and the human CD4 antigen with a truncated cytoplasmic domain (pMACS4.1; MilliMen Biotec) at a ratio of 3:1. CD4-positive cells were selected using magnetic beads coated with antibody specific for CD4 according to the manufacturer’s instruction (Dynabeads CD4; Invitrogen). Selected cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 7 μg/ml aprotinin, and 20 mM β-glycerophosphate), and α-tubulin, acetylated α-tubulin, and SIRT2 were detected by immunoblotting.

Plasmid constructs

All SIRT2 constructs and Cdk5 were generated by recombining the PCR-amplified ORF into pDON/FR201 of the Gateway cloning system (Invitrogen) by virtue of attB1 and attB2-mediated BP-clonase reaction. A subsequent LR-recombination reaction with Gateway-compatible pGex4T2 and pEVR0-HA resulted in GST- and HA-tagged expression constructs. Site-directed mutagenesis was performed using the quick change protocol (Stratagene) to generate the S331A, S331D, S331E, H150Y mutants. All constructs were verified by DNA sequencing. pCMV-EF1a-GFP/tubulin was cloned by standard procedures. For constructs expressing siRNA specific for SIRT2, the sequence 5′-GATCCCCGT GGATACG TCAAGTCCG TCTCAGGC CGCTGTGAAC3′ was cloned into pSUPER (provided by R. Bernards, Netherlands Cancer Institute, Amsterdam, Netherlands). This sequence is identical for both human and mouse. For a mouse-specific siSIRT2, the sequence 5′-GATCCCCCTT CACCGCGGCGG TCTCAGGTTCA AGAAGAGAAG GAGCGCGGT GGAAGTTTGG CCAAA-3′ was used. Additional constructs have been described previously (Rottmann et al., 2005; Luscher-Firzlaff et al., 2006).

Preparation of active cyclin–Cdk complexes

Expression and purification of human cyclin D, A, and E in complexes with Cdk2 and 4 from insect cells coinfected with recombinant baculoviruses were performed as previously described (Sarcevic et al., 1997). Baculoviruses expressing cyclin B and Cdk1 were provided by D. Morgan (University of California, San Francisco, San Francisco, CA). The activities of Cdk4 or Cdk2 and 1 complexes were measured using p2178-929 or histone
H1, respectively. 1 catal of cyclin-Cdk activity incorporates 1 mol of phosphate in 30 μl of kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂, 0.01% Tween-20, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM orthovanadate, 0.01% BSA, and 25 μM ATP) containing 5 μg pRb[76829] or histone H1 and 25 μM ATP. The incorporation of phosphate was linear over the incubation time of 30 min. The p35–Cdk5 complex was obtained from Millipore.

Solid phase phosphorylation
High-density protein arrays enriched for high expression of His-tagged proteins were obtained from the German Resource Center for Genome Research (HeiX library; http://www.rzpd.de). For phosphorylation cloning, filters were rehydrated and the cell debris was washed off twice in 20 mM Tris-Cl, pH 7.5, 0.5 M NaCl, and 0.5% Tween-20 for 10 min each. Further processing steps were three incubations in 20 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 20 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 3% BSA for 10 min each. Filters were briefly equilibrated in kinase buffer and then incubated in the same buffer at room temperature for 1 h. Next, the filters were subjected to phosphorylation by incubation in kinase buffer supplemented with 2.5 pcatal/ml cyclin E–Cdk2 and 200 kBq/ml [γ-32P]ATP at 30 °C for 90 min. Finally, filters were washed in 50 mM Hepes, pH 7.5, 10 mM MgCl₂, 250 mM NaCl, and 0.2 mM PMSF and exposed to x-ray film for autoradiography.

In vitro kinase assays, in vivo labeling, and phosphatase assays
In vitro phosphorylation of purified GST fusion proteins was performed as described previously (Sarcevic et al., 1997), except that incubations were at 30 °C for 30 min. For in vivo labeling, HEK293 cells were transiently transfected and, 2 d later, metabolically labeled in phosphate-free DME supplemented with 10% dialyzed FCS, 20 mM sodium bicarbonate, 18 mM Hepes, pH 7.5, and 10–20 MBq of [γ-32P]ATP for 2 h. The labeled cells were lysed in RIPA buffer. The HA-tagged SIRT2 proteins were immuno-precipitated with α-HA mAb (3F10) and protein G–agarose. The precipitates were washed four times in the same buffer and separated by SDS-PAGE on 12% gels (Luscher-Frizzall et al., 2006).

For phosphatase treatment, cells were lysed in RIPA buffer and SIRT2 was immunoprecipitated using polyclonal antisera and washed. Immobilized proteins were resuspended in 50 mM Tris-Cl, pH 8.5, and 1 mM MgCl₂. The probes were then incubated in the presence of 0.7 U of bacterial alkaline phosphatase (Sigma-Aldrich) at 30 °C for 30 min. The reactions were stopped by adding SDS sample buffer.

Phosphopeptide identification by nonliquid chromatography tandem mass spectrometry
Proteins were treated with DTT and iodoacetamide and digested in gel by trypsin. Before nonliquid chromatography tandem mass spectrometry analysis, all trypsin-digested samples were purified and desalted using C18 STAGE tips (Rappsilber et al., 2003).

Peptide identification experiments were performed using a nano-HPLC 1100 nano-flow system (Agilent Technologies) connected online to a 7-Tesla linear quadrupole ion trap–Fourier transform mass spectrometer (Thermo Fisher Scientific). Peptides were separated on 15 cm of 100 μm ID PicoTip columns (New Objective, Inc.) packed with 3 μm Reprosil C18 beads (Dr. Maisch, GmbH) using a 90-min gradient from 90% buffer A/10% buffer B to 65% buffer A/35% buffer B (buffer A contains 0.5% acetic acid and buffer B contains 80% octanethiol in 0.5% acetic acid) with a flow rate of 300 nl/min. Peptides eluting from the column tip were electrosprayed directly into the mass spectrometer with a spray voltage of 2.1 kV. The mass spectrometer was operated in the data-dependent mode to sequence the four most intense ions per duty cycle. In brief, full-scan mass spectrometry spectra of intact peptides (m/z 350–2,000) with an automatic gain control (AGC) accumulation target of 10⁵ ions were generated in the Fourier transform ion cyclotron resonance cell with a resolution of 50,000. The four most abundant ions were sequentially isolated and fragmented in the linear ion trap by applying collision-induced dissociation using an accumulation target value of 20,000 (capillary temperature, 200°C; normalized collision energy, 30%). A dynamic exclusion of ions previously sequenced within 180 s was applied. All unassigned charge states were excluded from sequencing. A minimum of 500 counts was required for mass spectrometry 2 selection. Data-dependent neutral loss scanning of phosphoric acid groups was enabled for each mass spectrometry 2 spectrum among the three most intense fragment ions.

RAW spectrum files were converted into a Mascot generic peak list by DTA Supercharge (http://msquant.sourceforge.net). Peptides and proteins were identified using the Mascot algorithm (Matrix Science) to search a local version of the DIP protein database (release 48.0). The following initial search criteria were applied: 20 parts per million (ppm) for the parent peptide and 0.5 D for fragmentation spectra and a fixed carbamidomethyl modification for cysteines. Oxidation of methionine, deamidation (glutamine and asparagine), and phosphorylation (serine, threonine, and tyrosine) were searched as variable modifications. Parent ion masses were internally calibrated by MSQuant (http://msquant.sourceforge.net) to obtain accurate masses better than 5 ppm. Fragmentation spectra of phosphopeptides were manually verified.

Antibodies, immunoprecipitation, and Western blotting
The polyclonal anti-SIRT2 T749 and 1809 antisera were raised against a bacterially expressed GST-SIRT2 fusion protein (Eurogentec). The following antibodies are commercially available from Abcam: rabbit anti-Cdk2 pab H9298, Cdk5 pab C-8, Cdk5 pab M-20, GFP pab FL, p27 Kip1 pab C-19, p35 pab C-19, and SIRT2 mAb A-5 (Santa Cruz Biotechnology, Inc.). HA-tag mAb 3F10 (Roche), and acetyl-α-tubulin mAb 6–11B-1 and α-tubulin mAb B-5-1-2 (Sigma Aldrich). Low stringency immunoprecipitations and coimmunoprecipitations and Western blotting were done as described previously (Vervoorts et al., 2003). mAbs specific for Ser=331 phosphorylated human SIRT2 were generated against the synthetic peptide NPSTSAS(phosphorylated)PKSSPPADEARTEKERPO in Lou/C rats.

HDAC assay
Immunoprecipitated material was washed in SIRT2 deacetylase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂, 10 mM NaF, 10 mM β-glycerophosphate, 0.2 mM DTT, and 25 μM ATP), which is identical to the kinase buffer. Both immunoprecipitated material and recombinant SIRT2 were resuspended in 50 μl SIRT2 deacetylase buffer containing 1 mM NAD⁺ (Sigma-Aldrich) and 5 Acetylated chicken reticulocyte core histone (~7.5 μg corresponding to 12,000 cpm per reaction; obtained from P. Loidl, Innsbruck Medical University, Innsbruck, Austria). The reactions were performed in the presence of 20 μM trichostatin A to block potentially copurified HDAC activities at 37°C for 60 min. The acetyl-group of O-acetyl-ADP-ribose was hydrolyzed by adding 15 μl of 1 N NaOH at room temperature for 20 min, followed by adding 185 μl of 0.1 M HCl and 0.16 M acetic acid. Released [3H]acetate was extracted in 750 μl ethyl acetate and counted (Borra et al., 2004). Deacetylation of brain-derived α-tubulin was performed with 25 μg of P3-P4 mouse forebrain lysate essentially as described previously (North et al., 2003).

Immunocytochemistry
 Cultures were fixed with PBS containing 4% paraformaldehyde and 0.5% sucrose for 15 min followed by washing and permeabilization in PBS with 0.1% Triton X-100 for 5 min. The samples were blocked in PBS with 2% BSA and incubated with primary antibodies for 1 h. Primary antibodies were used as follows: rabbit anti-SIRT2 [1:500], mouse anti-SIRT2 [1:50; Santa Cruz Biotechnology, Inc.], rabbit anti-p35 [1:50; Santa Cruz Biotechnology, Inc.], mouse anti-β-tubulin [1:1,500; Sigma-Aldrich], and mouse anti-acetylated α-tubulin [1:1,500]. Secondary antibodies conjugated to Alexa488, 546, or 660 [1:1,000; Invitrogen] were applied for 1 h along with Texas red phalloidin or DAPI to highlight filamentous actin. After washing with PBS and staining with DAPI for 5 min, coverslips were mounted with moldav. Images of cells were acquired at room temperature on a microscope (Axiovert; Carl Zeiss, Inc.) using either a 20x Plan-NeoFluar 0.5 NA [Carl Zeiss, Inc.] or a 63x Plan-NeoFluar 1.3 NA [Carl Zeiss, Inc.] lens without oil. A camera (AxioCam; Carl, Zeiss Inc.) was used with AxioVision 4.6 software [Carl, Zeiss, Inc.] for deconvolution and Photoshop CS (Adobe) for adjusting contrast and brightness was used.

Colocalization of SIRT2 and p35 expression was performed by analyzing z stacks using AxioVision 4.6 software. Fig. S5 D is a typical result obtained from one individual section of the z-stack series. Fields 1 and 2 contain pixels of SIRT2 and p35, respectively, not colocalizing, whereas area 3 reveals pixels overlapping area 4 contains largely background signals surrounding the cells; Fig. S5 D).

Neurite outgrowth and growth cone collapse assays
Acid-treated coverslips (diameter, 13 mm) were coated with 100 μg/ml poly-lysine (Sigma-Aldrich) in borate buffer at 37°C for 1 h, followed by washing in PBS. A culture with 20 μg/ml mouse laminin (Invitrogen) in HBSS at 37°C for 3–4 h. After additional washing steps, coverslips were kept in DME with 10% HS at 37°C until used. Neurons were plated at a density of 5 × 10⁴ – 2 × 10⁵ cells per coverslip and stained after 2 d of in vitro culture. For growth cone collapse assays, 2-d-old cultures were incubated at 37°C for 30 min with 1 μg/ml of preclustered ephrin-A5-Fc with 10 μg/ml anti-human IgG, Fc-specific, for 30 min (Sigma-Aldrich) or Fc alone followed by staining for F-actin and microtubules.


