SIRT1 phosphorylation and cell proliferation.

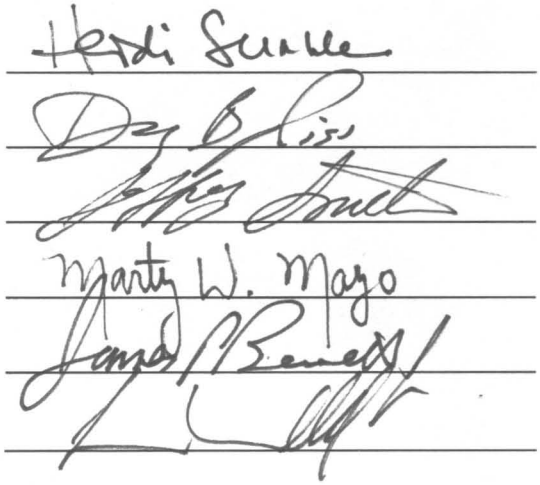
Tsutomu Sasaki
Tokyo, Tokyo, Japan

M.D., University of Tokyo, 1997

A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Neuroscience

University of Virginia
August, 2007



Abstract

SIRT1, the mammalian ortholog of Sir2, is an NAD^+-dependent deacetylase, and its gene expression can affect the lifespan of multiple organisms, as can compounds that pharmacologically activate it. It possesses a large and growing list of substrates, such as p53, NFκB, and FoxO, and influences cell survival, stress resistance, cell differentiation, glucose and fat metabolism. Importantly, SIRT1 may regulate longevity and has proved to be a significant factor in aging research. However several key questions have not been addressed, namely: 1) are SIRT1 levels regulated in the context of aging and senescence, and 2) is SIRT1 regulated post-translationally.

In order to address the first question, I investigated the changes in the level of SIRT1 with aging and senescence, and showed that levels of the SIRT1 protein decline as cells lose mitotic activity with age and that the decline is due to a post-transcriptional mechanism. These results are described in chapter 2.

Based on the above findings, I tested the hypothesis that SIRT1 is regulated post-translationally by phosphorylation conferred by cyclin-dependent kinase (Cdk) in chapter 3. I found that SIRT1 interacts with and is phosphorylated by cyclin B and Cdk1 *in vivo*. Furthermore, SIRT1 is a good substrate for cyclin B/Cdk1 *in vitro* and this complex phosphorylates SIRT1 at Thr530 and Ser540. These phosphorylation sites and recognition motifs for cyclin and Cdk are conserved among different species. Thus, SIRT1 is a substrate for cyclin B/Cdk1.

I also investigated how phosphorylation of SIRT1 affects its deacetylase activity. Treating SIRT1 with phosphatase reduces its NAD^+-dependent deacetylase activity. I further explored the biological functions of SIRT1 phosphorylation at Thr530 and Ser540 by using SIRT1 with a double mutation for T530A/S540A (AA mutant). Wild-type SIRT1, but not the AA mutant, rescued the slower growth rate and the deficit in S-phase cells, which were observed in $Sirt1^{-/-}$ ES cells. In summary, SIRT1 is phosphorylated by Cyclin B/Cdk1, and this process regulates its deacetylase activity and affects cell proliferation.

Thus SIRT1 is regulated in the context of aging and senescence by Cdk1 and in return controls cell proliferation.

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Abbreviations

ADP: adenosine diphosphate

AL: ad libitum

ANOVA: analysis of variance

APC: anaphase-promoting complex

ATM: ataxia-telangiectasia mutated

ATP: adenosine triphosphate

Cdk: cyclin-dependent kinase

CEBPα: CCAAT enhancer binding protein alpha

CIP: calf intestinal phosphatase

CK1: casein kinase 1

CK2: casein kinase 2

CR: caloric restriction

CtBP: C-terminal binding protein

DMEM: Dulbecco's modified Eagle medium

DNA-PK: DNA-dependent protein kinase

DTT: dithiothreitol

ERK1: extracellular signal-regulated kinase 1

ES cell: embryonic stem cell

FBS: fetal bovine serum

FoxO: forkhead box transcription factor O

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GHRKO: growth hormone-receptor knock-out

GST: glutathione S-transferase

GSK3: glycogen synthase kinase-3

HAT: histone acetyltransferase

HIC1: hypermethylated in cancer 1

HDAC: histone deacetylase

IC_{50}: inhibitory concentration 50%

IGF-1: insulin-like growth factor 1

IGF1R: IGF-1 receptor

IKK: I kappa B kinase

IP: immunoprecipitation

λppase: lambda protein phosphatase

MAPK: mitogen-activated protein kinase

MEF: mouse embryonic fibroblasts

mRNA: messenger RNA

MW: molecular weight

NAD: nicotinamide adenine dinucleotide

NEDD4: neural precursor cell expressed, developmentally down-regulated 4

NFκB: nuclear factor-kappa B

Nmnat: nicotinamide mononucleotide adenylytransferase

NT: non-transgenic

O/N: overnight

PBS: phosphate-buffered saline

PCNA: proliferating cell nuclear antigen

PDK: proline-dependent kinase

PGC1α: peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

PKA: protein kinase A

pre-RC: prereplication complex

PPAR-γ: peroxisome proliferator-activated receptor gamma

RIPA buffer: radioimmunoprecipitation assay buffer

RT-PCR: reverse transcription-polymerase chain reaction

SA-βGal: senescence-activated beta-galactosidase

SAC: spindle assembly checkpoint

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of margin

Ser: serine

SIR: silent information regulator

STAC: sirtuin activator

Thr: threonine

Tg: transgenic

TNFα: tumor necrosis factor alpha

UCP2: uncoupling protein 2

WB: western blot;

WT: wild-type

Chapter 1: Background and Significance

This dissertation entitled "SIRT1 phosphorylation and cell proliferation" explores two important questions, namely: 1) is SIRT1 regulated in the context of aging, and 2) is it regulated by cyclin-dependent kinases (Cdks) via phosphorylation. In this section, I will define SIRT1 and discuss its significance in aging research and how it affects aging and mammalian physiology through its substrates. Then I will discuss how SIRT1 and Cdks are regulated.

1.1 SIRT1 and aging

1.1.1 What is SIRT1?

SIRT1 is the mammalian homologue of SIR2 which is a Nicotinamide Adenine Dinucleotide $(NAD)^+$-dependent deacetylase (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000) that was first identified in yeast by screening silent mating-type loci mutants (Rine and Herskowitz 1987). Unlike *SIR3* or *SIR4*, which are also involved in silencing mating-type loci, the *SIR2* gene is highly conserved in organisms ranging from archea to humans (Brachmann *et al.* 1995). There are seven sirtuin (SIR2 family proteins) homologues in humans (SIRT1-7) (Frye 1999; Frye 2000). SIRT1 is the most closely related to *Saccharomyces cerevisiae* SIR2 (Frye 2000). The deacetylase activity of SIR2 is critical for extending the replicative lifespan of yeast (Lin *et al.* 2000). Moreover, overexpressing *SIR2* and its ortholog in yeast (Kaeberlein *et al.* 1999), worms (Tissenbaum and Guarente 2001) and flies (Rogina and Helfand 2004) extends their lifespan, an effect that can be reproduced by pharmacological activators of sirtuins

(STACs) (Howitz *et al.* 2003; Wood *et al.* 2004). One of the STACs, resveratrol, improved the health and survival of mice on a high-calorie diet by improving mitochondrial function and protecting against metabolic disease by activating SIRT1 and one of its substrates, PGC1α (Baur *et al.* 2006; Lagouge *et al.* 2006).

In mammalian cells, SIRT1 regulates the activity of various targets, such as p53 (Luo *et al.* 2001; Vaziri *et al.* 2001; Langley *et al.* 2002), nuclear factor-kappa B (NFκB) (Yeung *et al.* 2004), and forkhead (FoxO) (Brunet *et al.* 2004; Daitoku *et al.* 2004; Motta *et al.* 2004; van der Horst *et al.* 2004; Yang *et al.* 2005). In the case of p53, SIRT1 deacetylates and attenuates p53-mediated transcriptional activation of its target genes (Luo *et al.* 2001; Vaziri *et al.* 2001; Langley *et al.* 2002).

1.1.2 SIRT1, caloric restriction and lifespan extension

SIRT1 may regulate longevity of multiple organisms by mechanisms that are involved in caloric restriction (CR). Reducing the amount of calories ingested by 30-50% is the only regimen that is reported consistently to increase both the mean and maximum lifespan of various organisms, including non-human primates (Lane *et al.* 1996; Kealy *et al.* 2002; Roth *et al.* 2002; Richardson *et al.* 2004). CR also broadly slows the effects on aging in many physiological functions (Weindruch and Walford 1988), and protects against numerous diseases in rodent models, including cardiovascular disease, cancer, diabetes and neurodegenerative diseases (Fernandes *et al.* 1976; Ingram *et al.* 1987; Zhu *et al.* 1999); studies in primates, including humans, are in progress (Mattison *et al.* 2003; Fontana *et al.* 2004; Heilbronn *et al.* 2006).

The first experiments showing associations between SIRT1 and lifespan extension were performed in yeast. The initial link between *SIR2* and aging came from the finding that specific mutations in a component of the yeast Silent Information Regulator (SIR) complex (*SIR2*, *SIR3*, and *SIR4*) extended the replicative lifespan (Kennedy *et al.* 1995). Subsequent work by the same group demonstrated that *SIR2* alone can promote longevity in *S. cerevisiae*. They showed that loss-of-function mutations in *SIR2* shortened yeast lifespan by ~50%, and increasing *SIR2* gene expression extended it by ~30% (Kaeberlein *et al.* 1999). Moreover, mutations that abolish SIR2 deacetylase activity shorten the life of yeast (Imai *et al.* 2000). Therefore, SIR2 and its normally functioning deacetylase activity is important for longevity in yeast.

SIR2 may be important for the increased survivability associated with CR. Deleting *SIR2* abolished the ability of CR to extend lifespan in yeast (Lin *et al.* 2000; Lin *et al.* 2002) and silencing activity of SIR2 is increased with CR *in vivo* (Lin *et al.* 2002). In addition, an activator of the SIR2 enzyme, resveratrol, was shown to extend yeast replicative lifespan (Howitz *et al.* 2003). Despite this evidence, subsequent studies performed in other strains of yeast provided data inconsistent with the idea that CR activates SIR2 and that this activation results in a longer life. First, lifespan extension in response to CR is greater in cells lacking *SIR2* than in wild-type cells. This depends on the levels of extrachromosomal ribosomal DNA circles (ERC) (Sinclair and Guarente 1997), which can be suppressed by deleting the *FOB1* gene (Kaeberlein *et al.* 2004). Second, CR increases lifespan additively with either overexpression of *SIR2* or deletion of *FOB1* (Kaeberlein *et al.* 2004) and, third, *in vivo* SIR2 activity is not increased by CR,

as measured by transcriptional silencing of a subtelomeric marker gene (Kaeberlein *et al.* 2005; Kaeberlein *et al.* 2005). Fourth, respiration is not required for CR-induced lifespan extension (Kaeberlein *et al.* 2005), which is inconsistent with a previous model of how SIR2 might be activated by CR (Lin *et al.* 2004). Fifth, the ability of nicotinamide to prevent lifespan extension by CR in cells with reduced ERC accumulation is not due to SIR2 inhibition (Kaeberlein *et al.* 2005). Finally, it was recently reported that CR increases lifespan independently of all SIR2-family proteins in yeast (Tsuchiya *et al.* 2006). Therefore, yeast appears to have both SIR2-dependent and SIR2-independent components to CR-mediated longevity extension.

While the controversy over the role of SIR2 in yeast aging was developing, its role in aging of other organisms was investigated. The *SIR2* ortholog in *Caenorhabditis elegans* (*sir-2.1*) was shown to be a key determinant of lifespan in worms (Tissenbaum and Guarente 2001). Increased expression of the *sir-2.1* gene extends the lifespan of worms, where it functions upstream of *daf-16* in the insulin-like signaling pathway. The *sir-2.1* transgene and hypomorphic *daf-2* (Ins/IGF1R) allele does not work together to further extend the worm's life (Tissenbaum and Guarente 2001).

Several experiments in flies suggest that CR works by activating the *Drosophila melanogaster SIR2* ortholog, *dSir2*. CR increases the levels of *dSir2* mRNA in *Drosophila* (Rogina *et al.* 2002). Resveratrol, which activates *dSir2*, extends the life of the fly and this extension was not observed in *dSir2* mutants (Wood *et al.* 2004). Moreover, these mutants did not show an extended lifespan with CR (Rogina and Helfand 2004). Resveratrol did not further prolong the life of CR flies, indicating that

dSir2 and CR affect the same pathway. Overexpression of *dSir2* can also extend the fly lifespan, and it was not further increased by CR. These findings suggest that the role of *SIR2* as mediator of CR's effects on lifespan has been conserved in metazoans. However, whether or not its mammalian homologue, SIRT1, also decisively mediates the effects of CR is still unknown (Guarente and Picard 2005).

As mentioned above, resveratrol is a potent small molecule STAC that has been shown to increase longevity in multiple organisms, thus supporting a role of sirtuins in longevity. It extends lifespan by 70% in *S. cerevisiae* in the absence of stress; synergistic effects with CR were not observed (Howitz *et al.* 2003). Resveratrol and other STACs are effective in *C. elegans* and *D. melanogaster*, and extend their lifespan without reducing fecundity or feeding rate (Howitz *et al.* 2003; Wood *et al.* 2004). Resveratrol also improved the health and survival of mice on a high-calorie diet, by improving mitochondrial function and protecting against metabolic disease, which it did by activating SIRT1 and PGC1α (Baur *et al.* 2006; Lagouge *et al.* 2006).

In summary, SIRT1 orthologs and its pharmacological activators contribute to the mechanisms involved in CR-mediated longevity in multiple organisms.

1.2 Function of SIRT1 in mammalian physiology

In order to understand how SIRT1 may regulate longevity, it is important to understand its substrates and potential biological consequences of its actions on them in the context of mammalian physiology.

Unlike yeast SIR2, which has no known targets aside from histones, the mammalian SIR2 ortholog SIRT1 possesses a large and expanding list of targets (Blander and Guarente 2004). SIRT1 regulates various substrates, such as p53 (Luo *et al.* 2001; Vaziri *et al.* 2001; Langley *et al.* 2002), Bax (Cohen *et al.* 2004), NFκB (Yeung *et al.* 2004), and FoxO (Brunet *et al.* 2004; Daitoku *et al.* 2004; Motta *et al.* 2004; van der Horst *et al.* 2004; Yang *et al.* 2005), which results in attenuating cell death, promoting damage repair, and increasing oxidative stress resistance. It may also mediate at least some of the endocrine changes induced by CR in mammals, such as decreasing levels of growth hormone, insulin-like growth factor 1 (IGF-1), and thyroid stimulating hormone (Koubova and Guarente 2003). CR also increases insulin sensitivity and corresponding reductions in blood glucose and insulin levels (Barzilai *et al.* 1998; Dhahbi *et al.* 2001).

1.2.1 Stress resistance and cell survival

SIRT1 deacetylates important transcription factors, including p53 (Luo *et al.* 2001; Vaziri *et al.* 2001; Langley *et al.* 2002), FoxO (Brunet *et al.* 2004; Daitoku *et al.* 2004; Motta *et al.* 2004; van der Horst *et al.* 2004; Yang *et al.* 2005), NFκB (Yeung *et al.* 2004), and the DNA repair factor Ku (Cohen *et al.* 2004), thereby increasing the stress resistance of cells by inhibiting apoptosis and increasing repair. p53 is a crucial mediator of cellular responses to DNA damage, including the senescence response (Wahl and Carr 2001). Deacetylation of Lys382 by SIRT1 attenuates p53's transcriptional activity (Luo *et al.* 2001; Vaziri *et al.* 2001; Langley *et al.* 2002). FoxO controls the expression of various genes involved in regulating proliferation and survival of cells, such as cell cycle

proteins [*p27* (Medema *et al.* 2000), *cyclin B* and *polo-like kinase* (Alvarez *et al.* 2001), *cyclin G2* (Martinez-Gac *et al.* 2004)], DNA repair [*Gadd45a* (Tran *et al.* 2002)], oxidative stress response [*Sod2* (Kops *et al.* 2002), *Catalase* (Nemoto and Finkel 2002)], and apoptosis [*Bim* (Gilley *et al.* 2003), *FasL* (Kops and Burgering 1999; Ciechomska *et al.* 2003; Kavurma and Khachigian 2003)]. Deacetylation of FoxO by SIRT1 increases FoxO-induced cell cycle arrest and oxidative stress resistance, while inhibiting FoxO-induced cell death (Brunet *et al.* 2004). SIRT1 also deacetylates RelA/p65 at lysine 310, a site critical for NFκB transcriptional activity, thereby sensitizing cells to apoptotic death by the cytokine TNFα (Yeung *et al.* 2004). Furthermore, SIRT1 can inhibit stress-induced apoptotic cell death by deacetylating the DNA repair factor Ku70 which in turn causes sequestration of the pro-apoptotic factor Bax away from mitochondria (Cohen *et al.* 2004). More recent work has shown that upon exposure to radiation, SIRT1 can enhance DNA repair capacity by Ku70 deacetylation (Jeong *et al.* 2007).

The effects of SIRT1 on stress resistance and cell survival are exemplified by investigations into the *wld-s* strain of mice that display much slower axonal degeneration in response to peripheral neuron injury. This protective effect was due to increased intraneuronal activity of the NAD^+ biosynthetic enzyme, Nmnat1. Elevated NAD^+ levels were shown to protect against neuronal degeneration in wild-type mice in a SIRT1 dependent manner (Araki *et al.* 2004). SIRT1 may protect neuronal cells by increasing stress resistance. Although the above studies did not mention if FoxO deacetylation was involved, they did show that SIRT1 protects cells from stress.

SIRT1 has also been shown to promote cell survival in models of aging-related neurodegenerative diseases, namely polyglutamine diseases such as Huntington's disease. For example, in transgenic *C. elegans*, which express mutant polyglutamine and show early neuronal dysfunction, the phenotype was rescued by increasing *sir-2.1* expression or treating with resveratrol. Resveratrol also rescued neurodegeneration in cells derived from Huntington's disease knock-in mice (HdhQ111) that have a mutation in which the polyglutamine repeats are expanded (Parker *et al.* 2005). Furthermore, SIRT1 inhibits apoptosis induced by other factors. For example, it can deacetylate Smad7 to prevent cell death induced by transforming growth factor β in glomerular mesangial cells (Kume *et al.* 2006). SIRT1 can also deacetylate the tumor suppressor protein, p73, both *in vivo* and *in vitro*, which reduces its transcriptional activity thereby partly inhibiting p73-induced apoptosis in human cells (Dai *et al.* 2007).

1.2.2 Glucose homeostasis

Aging is associated with the progressive development of general insulin resistance (Miller and Allen 1973; Nyberg *et al.* 1976; DeFronzo 1981; Narimiya *et al.* 1984; Reaven *et al.* 1989; Bravo *et al.* 1996; Kumar *et al.* 1999; Blaak 2000; Gupta *et al.* 2000; Elahi *et al.* 2002). A critical component of the physiology of CR is increased insulin sensitivity and corresponding reductions in blood glucose and insulin levels (Barzilai *et al.* 1998; Dhahbi *et al.* 2001). Glucose homeostasis is maintained by the liver and pancreatic β-cells in response to changing nutrient conditions. Upon feeding, pancreatic β-cells sense a rise in serum glucose levels and secrete insulin, which promotes glucose

uptake and glycogen synthesis in the target tissues. Glucagon is secreted by pancreatic α-cells, and stimulates release of glucose from glycogen stores and *de novo* synthesis of glucose (gluconeogenesis) in the liver. SIRT1 positively regulates glucose-stimulated insulin secretion by pancreatic β-cells by repressing transcription of the mitochondrial uncoupling protein UCP-2 gene (Moynihan *et al.* 2005; Bordone *et al.* 2006). It also promotes the survival of pancreatic β-cells during oxidative stress (Kitamura *et al.* 2005).

During fasting, hepatocytes induce gluconeogenesis to supply other tissues with glucose. SIRT1 deacetylates and activates PGC1-α to coordinate the increase in expression of gluconeogenic genes with the repression of glycolytic ones, thereby increasing hepatic glucose output (Rodgers *et al.* 2005). In cultured hepatocytes, SIRT1 promoted FoxO1-dependent transcription of hepatic gluconeogenic genes upon stress (Frescas *et al.* 2005).

SIRT1 may also affect glucose homeostasis by regulating the response of target cells (such as muscle cells) to insulin (Guarente 2006). Insulin activates a pathway of intracellular kinases that regulate FoxO. Moreover, PGC1-α activates genes involved in mitochondrial biogenesis, fatty acid oxidation, and respiration. Treating mice with resveratrol, which activates Sirt1, significantly increased their aerobic capacity by decreasing PGC-1α acetylation and increasing PGC-1α activity. This in turn induces genes for oxidative phosphorylation and mitochondrial biogenesis. These effects of resveratrol were dependent on the presence of the *Sirt1* gene, the murine homologue of SIRT1 (Lagouge *et al.* 2006). In skeletal muscle, fasting induces PGC-1α deacetylation, which can be mediated by SIRT1. SIRT1 is required to activate mitochondrial fatty acid

oxidation genes and to induce and maintain fatty acid oxidation in response to low glucose concentrations (Gerhart-Hines *et al.* 2007). SIRT1 upregulation may contribute to the increased insulin sensitivity seen with CR (Bordone and Guarente 2005) via its effects on NFkB. SIRT1 attenuates the activity of NFkB (Yeung *et al.* 2004), which is a transcription factor that induces the expression of tumor necrosis factor (TNF) α in white adipose tissue (Yao *et al.* 1997). TNFα can render cells insulin resistant by down-regulating the synthesis of glucose transporters and interfering with insulin signaling (Stephens *et al.* 1997).

1.2.3 Regulation of adipocytes

In humans, age is a strong risk factor for fat accumulation (Toth and Tchernof 2000). Mice engineered to have less white adipose tissue live longer, even though they do not eat less (Bluher *et al.* 2003). SIRT1 promotes fat mobilization in white adipocytes by repressing PPAR-γ, which controls the expression of genes mediating fat storage. In differentiated fat cells, upregulation of SIRT1 triggers lipolysis and loss of fat (Picard *et al.* 2004). Overexpression of SIRT1 in a cell model of white adipocytes, 3T3-L1 cells, reduces adipogenesis and triglyceride accumulation in the lipid droplets of the cells. *In vivo*, fasting recruits SIRT1 to PPAR-γ responsive elements and promotes lipolysis by inhibiting PPAR-γ–mediated fatty acid trapping (Picard and Guarente 2005). SIRT1 also increases transcription of adiponectin, an adipose-derived hormone that plays an important role in maintaining energy homeostasis. SIRT1 elevates adiponectin

expression, which is diminished in both obesity and type 2 diabetes, by activating FoxO and increasing the interaction between FoxO1 and CEBPα (Qiao and Shao 2006).

1.2.4 Cell differentiation

As senescence of progenitor or stem cells could impair tissue renewal and function, SIRT1 may promote health and longevity by preventing stem cell and progenitor pools from differentiating prematurely. SIRT1 regulates the activity of MyoD (Fulco *et al.* 2003) and PPARγ (Picard *et al.* 2004), which stop cells from differentiating into myocytes and adipocytes, respectively. Overexpression of SIRT1 retards muscle differentiation, and cells with decreased SIRT1 differentiate prematurely (Fulco *et al.* 2003). It was also shown that SIRT1 overexpression blocks differentiation of cells into adipocytes by suppressing PPARγ activity (Picard *et al.* 2004).

1.2.5 SIRT1 substrates and aging

Although the list of SIRT1 substrates is expanding, I would like specifically to mention the contribution of two SIRT1 substrates, FoxO and p53, to the regulation of longevity.

Daf-16/FoxO transcription factors are downstream targets of insulin/IGF1 signaling, and mutations in this signaling pathway increase longevity in a FoxO-dependent manner (Kenyon *et al.* 1993; Lin *et al.* 1997; Ogg *et al.* 1997). This pathway was first linked to lifespan in *C. elegans*, where mutations in *daf-2*, a regulatory gene encoding an insulin/IGF1 receptor ortholog (Kimura *et al.* 1997), were found to double

the lifespan of worms (Kenyon *et al.* 1993). Insulin/IGF1 receptor mutations can also increase the lifespan of *Drosophila* by as much as 80% (Tatar *et al.* 2001). In addition, mutations in *chico*, a downstream insulin receptor substrate-like signaling protein (Clancy *et al.* 2001; Tu *et al.* 2002) and FoxO overexpression prolong lifespan (Giannakou *et al.* 2004; Hwangbo *et al.* 2004).

Unlike worms and flies, which have a single insulin/IGF1-like receptor, mice and humans have separate receptors for insulin and IGF1. Female mice haplo-insufficient for the IGF1 receptor (*Igf1r*+/-) live 33% longer than wild-types, and males live 16% longer (Holzenberger *et al.* 2003). Long-lived *Igf1r*+/- mice do not develop dwarfism, their energy metabolism is normal, and their nutrient uptake, physical activity, fertility and reproduction are unaffected. These mice also display greater resistance to oxidative stress (Holzenberger *et al.* 2003). In addition, mice that lack the insulin receptor in adipose tissue (FIRKO mice) live ~18% longer than wild-types. At 10 months of age, glucose tolerance was maintained in FIRKO mice but it was impaired in wild-type mice as a result of the insulin resistance that normally occurs with age (Bluher *et al.* 2002). Mutations in upstream genes that regulate insulin and IGF1 also extend lifespan by ~50%. Growth hormone receptor mutants (Coschigano *et al.* 2003) and the Ames and Snell dwarf mice, which have pituitary defects resulting in low levels of growth hormone and IGF1 (Brown-Borg *et al.* 1996; Flurkey *et al.* 2002), live longer than their respective wild-types.

The insulin/IGF1 pathway influences lifespan in worms, flies, and mammals [reviewed in (Tatar *et al.* 2003)]. Insulin/IGF-1 signaling functions exclusively during

adulthood to influence the lifespan of *C. elegans* (Dillin *et al.* 2002). As the insulin/IGF-1 signaling is important for growth until adulthood, it may be possible to promote the health and lifespan of organisms by inhibiting this signaling pathway, once development is completed. Since CR during adulthood effectively promotes longevity (Weindruch and Walford 1982), it may do so, in part, by regulating the insulin/IGF1 pathway via SIRT1.

One protein that modulates the aging process partly via the IGF1 pathway is p53, well known for its role in DNA damage repair, cell cycle arrest, and apoptosis. The importance of p53 in the regulation of aging has been established in a series of recent papers. Aging phenotypes are accelerated in genetically engineered mutant mice that express low levels of either artificially truncated p53 protein (*m* allele) (Tyner *et al.* 2002) or modest overexpression of the naturally occurring short isoform of the truncated p53 protein (p44) (Maier *et al.* 2004). The truncated p53 protein forms a tetramer with full-length p53 and functions to modulate its activity as a transcription factor. This process results in cell cycle arrest accompanied by premature cellular senescence and hyperactivity of the IGF1 pathway, but does not increase apoptosis (Maier *et al.* 2004). On the other hand, "Super p53" mice, which carry a bacterial artificial chromosome (BAC) transgenic allele of p53 in addition to the two endogenous alleles, do not show any indication of premature aging; they exhibit an enhanced response to DNA damage and are significantly protected from cancer compared to normal mice (Garcia-Cao *et al.* 2002). In these mice, the ratio between the short isoform and full length p53 is not altered. Thus, dysregulation of p53 function, due to an altered ratio between short

isoform and full-length p53, is the key to accelerated aging phenotypes and premature cell senescence.

1.3 Molecular regulation of SIRT1

The previous section discussed how SIRT1 regulates various physiological functions involved in longevity. This section will focus on how SIRT1 function, expression, and localization are regulated.

1.3.1 Regulation of SIRT1 deacetylase activity

SIRT1 promotes the reaction between NAD^+ and acetylated proteins in which the protein is deacetylated and O-acetyl-ADP-ribose and nicotinamide are formed. Because SIRT1 deacetylase activity is dependent on NAD^+, SIRT1 functions as an energy sensor (Guarente 2000; Imai *et al.* 2000) or redox sensor (Fulco *et al.* 2003) that links energy metabolism to transcriptional regulation via deacetylation of histones, transcription factors, and co-factors. In yeast, when nutrients are scarce, cells preferentially employ respiration (oxidative phosphorylation) rather than fermentation to generate ATP (Lin *et al.* 2002). This process results in reducing NADH, acting as an electron donor for respiration (Lin *et al.* 2004), thus increasing NAD^+/NADH ratio, and/or decreasing levels of the SIR2 inhibitor nicotinamide. These actions in turn activate SIR2, increase rDNA stability (Blander and Guarente 2004), and extend lifespan. This metabolic shift may be analogous to a known transition that occurs in mammals during CR. Muscle cells switch from using glucose, which is metabolized in fed animals, in part fermentatively

(producing lactate), to utilizing fatty acids, which are metabolized oxidatively (Guarente and Picard 2005). Lactate decreases the ratio of NAD^+/NADH, while pyruvate increases it, and regulates SIRT1 activity (Fulco *et al.* 2003).

NAD^+ biosynthesis in vertebrates is markedly different from that of yeast and invertebrates (**Fig. 1**, from Revollo *et al.*) (Revollo *et al.* 2004). In yeast, increased activity of the *PNC1* gene product (nicotinamidase) improves nicotinamide clearance and extends its replicative lifespan (Anderson *et al.* 2003; Gallo *et al.* 2004). In mammals, nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme in the NAD^+ biosynthesis pathway (Revollo *et al.* 2004). Recently, nicotinamide riboside was reported to promote silencing activity of SIR2 and extend lifespan in yeast via NRK and URH1/PNP1/MEU1 pathways, which convert nicotinamide riboside to NAD^+ (Belenky *et al.* 2007).

Although it has been suggested that nicotinamide plays a critical role as an endogenous inhibitor of SIR2 in yeast (Anderson *et al.* 2003; Gallo *et al.* 2004), its intracellular concentration in mammalian cells is likely below the IC_{50} values reported for SIR2 family members, which are 40-50 μM for human SIRT1 (Bitterman *et al.* 2002; Marcotte *et al.* 2004), and 130 μM for yeast HST2 (Schmidt *et al.* 2004). Instead, nicotinamide could promote SIRT1 activity in mammals by acting as a substrate for NAD^+ biosynthesis mediated by Nampt (Revollo *et al.* 2004). It has long been known that administering nicotinamide to mammals increases NAD^+ levels in tissues such as liver and kidney (Kaplan *et al.* 1956; Greengard *et al.* 1964; Revollo *et al.* 2004). NADH was previously shown to be a competitive inhibitor of NAD^+ *in vitro* (Lin *et al.* 2004),

but the high binding constant for NADH (mM) indicates that cellular NADH levels are unlikely to regulate SIRT1 activity under most physiological conditions (Schmidt *et al.* 2004).

Resveratrol, a pharmacological activator of SIRT1, is a polyphenol found in grapes and grape products and has the ability to scavenge free radicals generated by oxidation [discussed in (Borra *et al.* 2005)]. Of all small molecule activators of sirtuins, resveratrol is the most potent (Howitz *et al.* 2003). However, recent reports state that resveratrol is a substrate-specific activator of yeast SIR2 and human SIRT1. Resveratrol enhances binding and deacetylation of peptide substrates that contain *Fluor-de Lys*, a non-physiological fluorescent moiety used to screen for pharmacological sirtuin activators *in vitro*. However, it has no effect on peptides that lack the fluorophore (Borra *et al.* 2005; Kaeberlein *et al.* 2005). Although, the mechanism by which SIRT1 is activated by resveratrol is unclear, it has been shown to extend lifespan in yeast, worms and flies (Howitz *et al.* 2003; Wood *et al.* 2004), and to improve the health of mice on high-calorie diet in a SIRT1-dependent manner (Baur *et al.* 2006; Lagouge *et al.* 2006).

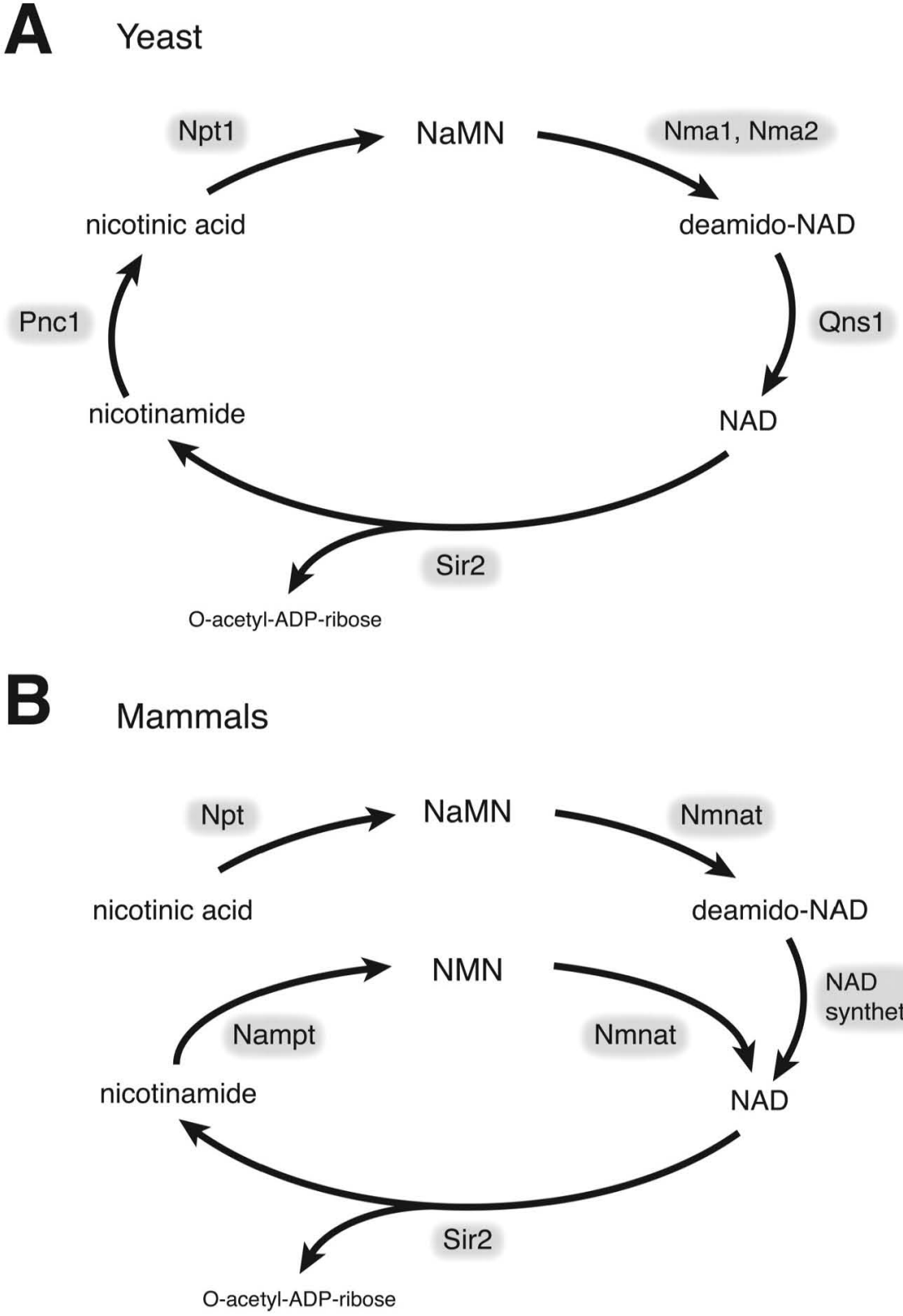

A
Yeast
Npt1
NaMN
Nma1, Nma2
nicotinic acid
deamido-NAD
Pnc1
Qns1
nicotinamide
NAD
Sir2
O-acetyl-ADP-ribose
B
Mammals
Npt
NaMN
Nmnat
nicotinic acid
deamido-NAD
NMN
NAD
synthetase
Nampt
Nmnat
nicotinamide
NAD
Sir2
O-acetyl-ADP-ribose

Figure 1. Differences in the NAD biosynthetic pathway between yeast (A) and mammals (B)

A. NAD biosynthesis from nicotinamide in *S. cerevisiae* is depicted. Pnc1, Npt1, Nma1, Nma2, and Qns1 are nicotinamidase, nicotinic acid phosphoribosyltransferase, nicotinic acid mononucleotide adenylyltransferase 1 and 2, and NAD synthetase, respectively. This pathway is also conserved in *C. elegans*, *Drosophila*, and other invertebrates.

B. NAD biosynthesis from nicotinamide and nicotinic acid in mammals is shown. These pathways are also conserved throughout vertebrates. Nicotinamide is the main precursor for NAD biosynthesis in mammals.

Abbreviations: NaMN, nicotinic acid mononucleotide ; Nampt, nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; Nmnat, nicotinamide/nicotinic acid mononucleotide adenylyltransferase; and Npt, nicotinic acid phosphoribosyltransferase.

From (Revollo *et al.* 2004). Refer to section 1.3.1 for a detailed descrirption.

1.3.2 Regulation of SIRT1 expression, turnover, and modifications

The SIRT1 gene is located on chromosome 10q21.3, and consists of 9 exons in humans (Voelter-Mahlknecht and Mahlknecht 2006). SIRT1 levels are modulated by CR or nutrient withdrawal, the mechanisms of which remain controversial. However, several studies suggest that SIRT1 protein levels are regulated at multiple stages of processing, namely at the transcriptional, post-transcriptional, and post-translational levels.

Transcriptional control of SIRT1 expression has been described in both flies and mammals. Levels of *dSir2* mRNA were increased by CR in *D. melanogaster* (Rogina *et al.* 2002). Similarly, mice subjected to an overnight fast showed increased *Sirt1* mRNA (and protein) levels in numerous tissues including skeletal muscle and liver (Nemoto *et al.* 2004). In mammalian cells, acute nutrient withdrawal (deprivation of glucose and serum) from cell cultures simultaneously activated FoxO3a, which in turn increased *SIRT1* expression. This effect was mediated through two p53 binding sites on the SIRT1 promoter, indicating that the release of p53 mediated transcriptional repression was involved.

Other mediators of the transcriptional control of SIRT1 expression include E2F1 and HIC1. E2F1 positively regulates SIRT1 transcription, and the increase in SIRT1 transcription in S-phase of the cell cycle correlates with elevated E2F1 (Wang *et al.* 2006). In contrast, the tumor suppressor HIC1 negatively modulates SIRT1 expression by forming a transcriptional repression complex with SIRT1 deacetylase. This complex directly binds the SIRT1 promoter and suppresses its transcription (Chen *et al.* 2005). SIRT1 expression can be increased by inhibiting the association between HIC1 and the

redox sensor CtBP using glycolytic blocker 2-deoxyglucose. Thus, HIC1:CtBP co-repressor complex is proposed to regulate SIRT1 specifically in response to nutrient deprivation (Zhang *et al.* 2007).

SIRT1 protein level is also regulated by humoral factors, namely insulin and IGF-1. SIRT1 was induced in CR rats and serum from these rats was used to trigger SIRT1 expression in human cells. This latter treatment caused reduced cell proliferation, enhanced tolerance to oxidants and heat, and heightened expression of stress-response genes (Cohen *et al.* 2004). CR serum contains decreased levels of glucose, triglycerides, free fatty acids, insulin, and IGF-1 compared to serum from animals fed ad-libitum (AL) (de Cabo *et al.* 2003). When CR serum was supplemented with insulin and IGF-1 to levels comparable to AL rat serum, the proliferative and stress-response phenotype seen in cells cultured with CR serum was attenuated, and SIRT1 protein level returned to normal (Cohen *et al.* 2004). Therefore, insulin and IGF-1 may be involved, at least in part, in the CR-induced increase in SIRT1 expression and its consequences on cell proliferation.

Another study reported that during fasting SIRT1 protein levels are regulated at the post-transcriptional level. Upon fasting in mice, levels of SIRT1 protein, but not mRNA, were increased. This may be mediated by pyruvate in the liver as pyruvate and glucose, but not insulin, regulated SIRT1 protein levels in cultured hepatocytes (Rodgers *et al.* 2005). SIRT1 protein synthesis was notably elevated by pyruvate, though pulse-radiolabeled SIRT1 protein levels did not change after 6 and 12 hours of pyruvate

treatment, indicating that a post-transcriptional mechanism is involved in regulating SIRT1 protein level.

Recently, another mechanism for post-transcriptional regulation of SIRT1 expression was reported. The RNA binding protein HuR, which regulates the stability of many target mRNAs, was associated with the 3' untranslated region of *SIRT1* mRNA and found to stabilize it, thereby increasing the level of SIRT1 protein. Oxidative stress triggered the dissociation of the [HuR-*SIRT1* mRNA] complex, in turn promoting *SIRT1* mRNA decay, reducing SIRT1 abundance, and lowering cell survival (Abdelmohsen ***et al.*** 2007).

Unfortunately, mechanisms underlying how the SIRT1 protein is degraded have not been elucidated. To date, all that is known is that SIRT1 can be cleaved by caspase during apoptosis (Ohsawa and Miura 2006). However, given the similarities between SIRT1 and SIRT2, which is a cytoplasmic isoform of SIRT1, examining how SIRT2 is modulated is informative. Levels of SIRT2 increase dramatically during mitosis and the protein is phosphorylated at multiple sites during the G2/M transition of the cell cycle. SIRT2 is dephosphorylated and its levels are subsequently decreased with overexpression of the protein phosphatase Cdc14b. The 26S proteasome may negatively regulate SIRT2 levels since inhibitors of this proteasome added to human cells (Saos2) increase SIRT2 protein levels (Dryden ***et al.*** 2003).

Post-translational modification of the SIRT1 protein has not been investigated extensively. Thus far, the only reported modifications are phosphorylation of serine 27 and serine 47 of human SIRT1, which were detected by screening nuclear lysates of

HeLa cells for phosphoproteins using mass spectrometry (Beausoleil *et al.* 2004). However, post-translational modifications of other deacetylases have been reported. There are three protein families with histone deacetylase (HDAC) activity: the SIR2 family of NAD^+-dependent HDACs (also called sirtuins), and the classical HDAC family (de Ruijter *et al.* 2003), members of which fall into two different classes, namely class I (HDAC 1-3, and 8) and class II (HDAC 4-7, 9, and 10). Both class I and II HDACs appear to be regulated via phosphorylation. For example, in human and murine cells, the HDAC1 protein is phosphorylated by PKA and CK2 *in vitro* (Cai *et al.* 2001) and HDAC3 is phosphorylated by c-Src (Longworth and Laimins 2006). HDAC1 (Pflum *et al.* 2001) and HDAC2 (de Ruijter *et al.* 2003) phosphorylation promotes enzymatic activity and complex formation. In addition, HDAC4, HDAC5 and HDAC7 interact with the 14-3-3 protein in a phosphorylation-dependent manner (Grozinger and Schreiber 2000) and phosphorylation of HDAC7 is regulated by multi-site hierarchical phosphorylation by a variety of kinases (Dequiedt *et al.* 2006). Although it is not known if SIRT1 function is regulated by phosphorylation, the available literature on HDACs is consistent with the idea that deacetylase function is regulated by phosphorylation, possibly by multi-site hierarchical phosphorylation.

1.3.3 Regulation of SIRT1 localization

SIRT1 was identified as a nuclear protein, but its cytoplasmic localization has been reported in murine pancreatic α- and β-cells and neonatal rat cardiomyocytes (Moynihan *et al.* 2005; Chen *et al.* 2006). Tanno *et al.* investigated the nucleo-

cytoplasmic relocation of SIRT1, and found that leptomycin B, an inhibitor of CRM1-mediated nuclear exportation, inhibited the shuttling. They identified two nuclear localization signals (amino acids 31-38 and 223-230 in mice, corresponding to 32-39 and 231-238 in humans) and two nuclear export signals (amino acids 139-145 and 425-431 in mice, corresponding to 146-153 and 433-439 in humans) (Tanno *et al.* 2007). However, the biological significance of SIRT1 shuttling between the nucleus and cytoplasm is unclear.

1.4 Cell cycle and the regulation of cyclin B/Cdk1 complex

Because this dissertation focuses on how SIRT1 modulates (and is modulated by) cell proliferation, in this section I will discuss how cyclin/Cdks are regulated and how they regulate the cell cycle.

1.4.1 Molecular mechanisms of the cell cycle

The cell cycle is regulated by combinations of cyclins and Cdks, and each cyclin/Cdk complex functions in a specific phase of the cell cycle (**Fig. 2**). In mammalian cells, the cell cycle machinery that determines whether cells will continue to proliferate, or cease division and differentiate, appear to operate mainly in the G_1 phase (Cheng 2004). There are two families of G_1 cyclins, namely, D and E cyclins (Miele 2004). In somatic cells, movement through G_1 into S phase is driven by the active form of the Cyclin D/Cdk 4, 6 complex, and subsequent phosphorylation of the Rb protein (Classon and Harlow 2002). At late G_1 phase, the Cyclin E/Cdk 2 complex further

propels cells into S phase, when DNA replication takes place. Cyclin A starts to accumulate during S phase, and is destroyed during mitosis, by proteasome-mediated degradation prior to metaphase (Yam *et al.* 2002). Cyclin A/Cdk2 phosphorylates E2F/DP complexes and inactivates them, preventing DNA replication from resuming and assuring only one replication of the genome during S phase. Cyclin B/Cdk1 propels cells into mitosis (M phase).

Cdk activity is strictly dependent on cyclin levels (Cheng 2004). In addition to regulation by cyclins and phosphorylation of their catalytic subunits, Cdks are largely controlled by endogenous Cdk inhibitors (Sherr and Roberts 1999). The Cip/Kip family of Cdk inhibitors ($p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$) may interact with a broad range of cyclin-Cdk complexes, while the INK4 family ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$) specifically inhibits Cdk4 and Cdk6.

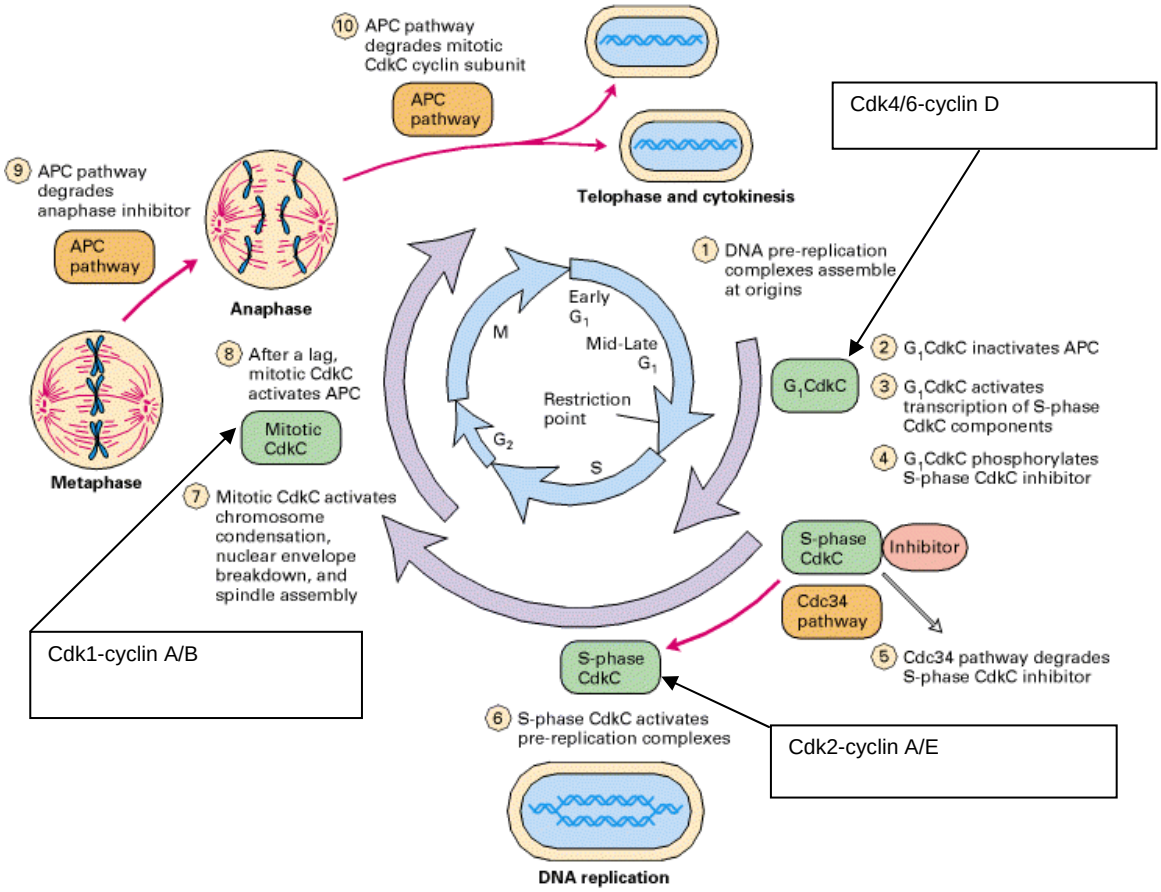

10 APC pathway degrades mitotic CdkC cyclin subunit
APC pathway
Telophase and cytokinesis
Cdk4/6-cyclin D
9 APC pathway degrades anaphase inhibitor
APC pathway
Anaphase
1 DNA pre-replication complexes assemble at origins
Early G1
Mid-Late G1
M
Restriction point
G2
S
2 G1CdkC inactivates APC
3 G1CdkC activates transcription of S-phase CdkC components
4 G1CdkC phosphorylates S-phase CdkC inhibitor
G1CdkC
8 After a lag, mitotic CdkC activates APC
Mitotic CdkC
Metaphase
7 Mitotic CdkC activates chromosome condensation, nuclear envelope breakdown, and spindle assembly
S-phase CdkC
Inhibitor
Cdc34 pathway
5 Cdc34 pathway degrades S-phase CdkC inhibitor
Cdk1-cyclin A/B
S-phase CdkC
6 S-phase CdkC activates pre-replication complexes
Cdk2-cyclin A/E
DNA replication

Figure 2. The cell cycle and Cdk/cyclins

From Molecular Cell Biology 4th edition, with modification. Refer to section 1.4.1 for a detailed description.

1.4.2 M phase events

In a typical somatic cell cycle, the M phase comprises mitosis and cytokinesis (reviewed in (Nigg 2001)). The main purpose of mitosis is to segregate sister chromatids into two nascent cells, such that each daughter cell inherits one complete set of chromosomes. In addition, each daughter cell must receive one centrosome and the appropriate complements of cytoplasm and organelles.

Mitosis is usually divided into five distinct stages: prophase, prometaphase, metaphase, anaphase and telophase (**Fig. 3**, from (Nigg 2001)). Cytokinesis, the process of cell cleavage, occurs at the end of mitosis. During prophase, interphase chromatin condenses into well-defined chromosomes and previously duplicated centrosomes migrate apart, thereby defining the poles of the future spindle apparatus. Concomitantly, centrosomes begin nucleating highly dynamic microtubules that probe space in all directions, and the nuclear envelope breaks down. During prometaphase, microtubules are captured by kinetochores (specialized proteinaceous structures associated with centromere DNA on mitotic chromosomes). Chromosomes then congregate on an equatorial plane, the metaphase plate, where they continue to oscillate throughout metaphase. After all the chromosomes have undergone a proper bipolar attachment of microtubules, a sudden loss of sister-chromatid cohesion triggers the onset of anaphase. Sister chromatids are then pulled towards the poles (anaphase A) and the poles themselves separate further towards the cell cortex (anaphase B). Once the chromosomes have arrived at the poles, nuclear envelopes reform around the daughter chromosomes,

and chromatin decondensation begins (telophase). Finally, an actomyosin-based contractile ring is formed and cytokinesis is completed.

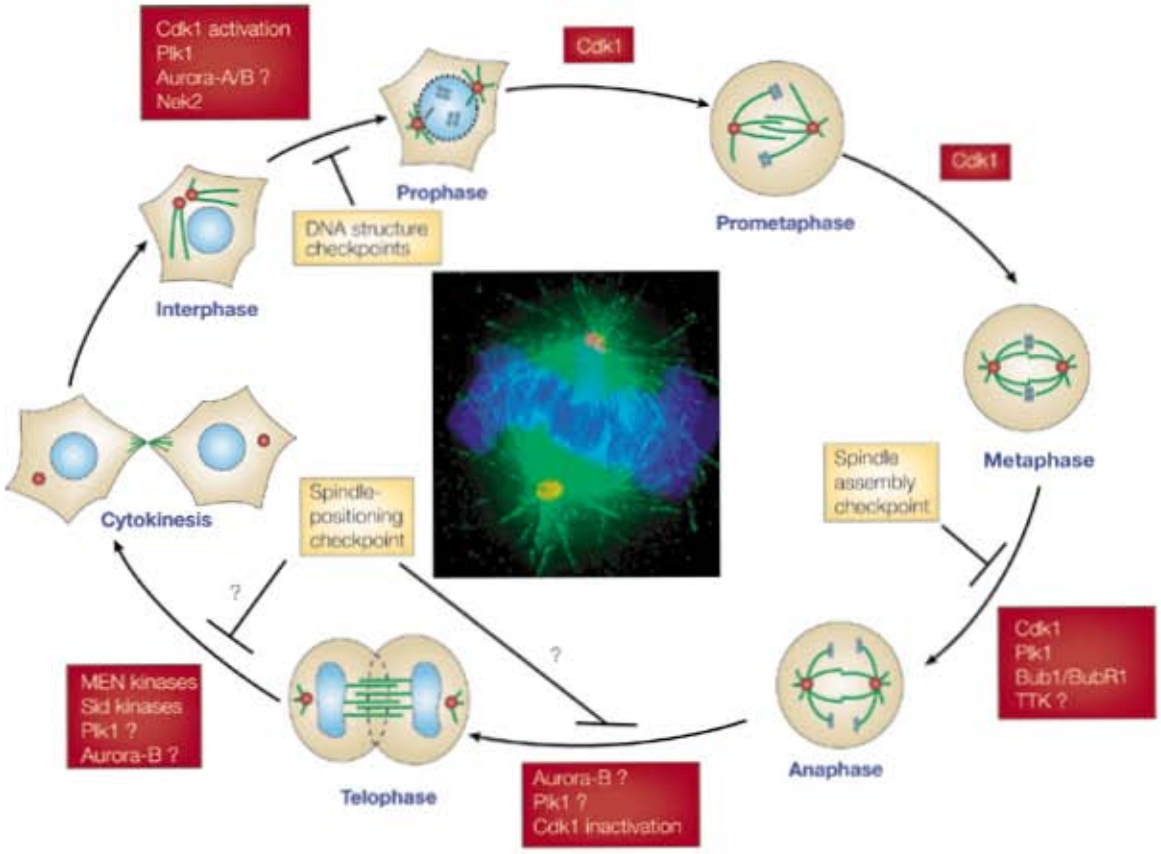

Cdk1 activation
Plk1
Aurora-A/B ?
Nek2
Cdk1
Prophase
Prometaphase
Cdk1
DNA structure checkpoints
Interphase
Metaphase
Spindle assembly checkpoint
Cytokinesis
Spindle-positioning checkpoint
?
?
Cdk1
Plk1
Bub1/BubR1
TTK ?
MEN kinases
Sid kinases
Plk1 ?
Aurora-B ?
Anaphase
Telophase
Aurora-B ?
Plk1 ?
Cdk1 inactivation
Nature Reviews | Molecular Cell Biology

Figure 3. Five stages of M-phase

The principal events typical of animal cell division can briefly be summarized as follows. During 'prophase', interphase chromatin condenses into well-defined chromosomes and previously duplicated centrosomes migrate apart, thereby defining the poles of the future spindle apparatus. Concomitantly, centrosomes begin nucleating highly dynamic microtubules that probe space in all directions, and the nuclear envelope breaks down. During 'prometaphase', microtubules are captured by kinetochores (specialized proteinaceous structures associated with centromere DNA on mitotic chromosomes). Although monopolar attachments of chromosomes are unstable, the eventual interaction of paired sister chromatids with microtubules emanating from opposite poles results in a stable, bipolar attachment. Chromosomes then congress to an equatorial plane, the metaphase plate, where they continue to oscillate throughout 'metaphase', suggesting that a balance of forces keeps them under tension. After all the chromosomes have undergone a proper bipolar attachment, a sudden loss in sister-chromatid cohesion triggers the onset of 'anaphase'. Sister chromatids are then pulled towards the poles (anaphase A) and the poles themselves separate further towards the cell cortex (anaphase B). Once the chromosomes have arrived at the poles, nuclear envelopes reform around the daughter chromosomes, and chromatin decondensation begins ('telophase'). Finally, an actomyosin-based contractile ring is formed and 'cytokinesis' is completed. The figure summarizes the stages of M phase. It also indicates where the major checkpoints exert quality control over mitotic progression and where mitotic kinases are thought to act. The insert illustrates a Ptk2 cell in metaphase; DNA is shown in blue (DAPI staining),

microtubules in green and spindle poles (-tubulin) in orange. (Picture kindly provided by P. Meraldi.) From (Nigg 2001).

1.4.3 Regulation of cyclin B/Cdk1 activity by mitotic checkpoint machinery

The cyclin B/Cdk1 complex was originally defined as the maturation-promoting factor or M phase-promoting factor (MPF), identified in meiotic frog eggs as a factor capable of inducing M phase in immature G_2 oocytes (reviewed in (Smits and Medema 2001)). During the normal cell cycle, negative regulation of cyclin B/Cdk1 at multiple levels prevents premature mitotic entry prior to completion of S phase (O'Farrell 2001). The cyclin B/Cdk1 complex is kept inactive by phosphorylation on tyrosine 15 and threonine 14 of Cdk1 by the kinases Wee1 and Myt1, respectively (Taylor and Stark 2001). In vertebrates, the rapid activation of cyclin B/Cdk1 during the terminal phase of G_2 commits the cells to mitosis, ultimately leading to the breakdown of the nuclear envelope (Pines and Rieder 2001). The Cyclin B/Cdk1 complex is activated largely through a combination of Wee1 inactivation and dephosphorylation of inhibitory phosphorylations on Cdk1 catalyzed by the Cdc25c phosphatase (O'Farrell 2001). Once activated, cyclin B/Cdk1 phosphorylates and inactivates its own inhibitors Wee1 and Myt1, whereas it also phosphorylates and activates its own activator Cdc25c (**Fig. 4**, from (Taylor and Stark 2001)).

There are two non-reversible transitions during mitosis, both of which are guarded by checkpoints. These checkpoints delay cells from entering and exiting mitosis under conditions that can compromise genome integrity. The first is the prophase-to-prometaphase transition, which culminates with the breakdown of the nuclear envelope. This defines the G_2-to-M transition, and it is regulated by checkpoints that operate during late G_2. The G_2/M checkpoint works by delaying activation of the master mitotic

regulator, cyclin B/Cdk1. Damage to DNA or the presence of unreplicated DNA prevents cyclin B/Cdk1 activation by both p53-dependent and -independent mechanisms (**Fig. 4**) (Miele 2004). DNA damage triggers phosphorylation of Cdc25c by Chk1 and Chk2, which exports Cdc25c to the cytoplasm. Three transcriptional targets of p53, Gadd45, p21, and 14-3-3σ inhibit cyclin B/Cdk1 activity. Gadd 45 promotes dissociation of cyclin B and Cdk1, while p21 can form complex with cyclin B/Cdk1 and block its enzymatic activity. Cyclin B1 is initially localized in the cytoplasm during S and G_2 phase, and translocates to the nucleus at the beginning of mitosis (Pines and Hunter 1991). 14-3-3σ sequesters the cyclin B/Cdk1 complex in the cytoplasm. Cdk1 can also be inhibited by p27 (Toyoshima and Hunter 1994). Both the *Cyclin B1* and *cdk1 (cdc2)* genes are transrepressed by p53 (Taylor and Stark 2001).

The second transition is the metaphase-to-anaphase transition, which leads to the disjunction of sister chromatids and exit from mitosis. This transition is regulated by pathways operating during mitosis that are defined primarily by the spindle assembly checkpoint (SAC). SAC prevents the activation of anaphase-promoting complexes (APC), which promotes proteolysis of securin and cyclin B, and allows chromatid separation (anaphase) and the cells to exit mitosis (telophase). SAC blocks cells from entering anaphase with misaligned chromosomes (Smits and Medema 2001; Morrison and Rieder 2004; Rieder and Maiato 2004). Once sister chromatid separation is triggered by destruction of the anaphase inhibitors, spindle disassembly and mitotic exit are initiated by degrading mitotic cyclins (Smits and Medema 2001). Cyclin B1 destruction starts as soon as the last chromosomes are aligned on the metaphase plate, and most is

degraded by the end of metaphase (Clute and Pines 1999). Upon cyclin destruction, Cdk1 is inactivated, setting the stage for mitotic exit and cytokinesis (Nigg 2001).

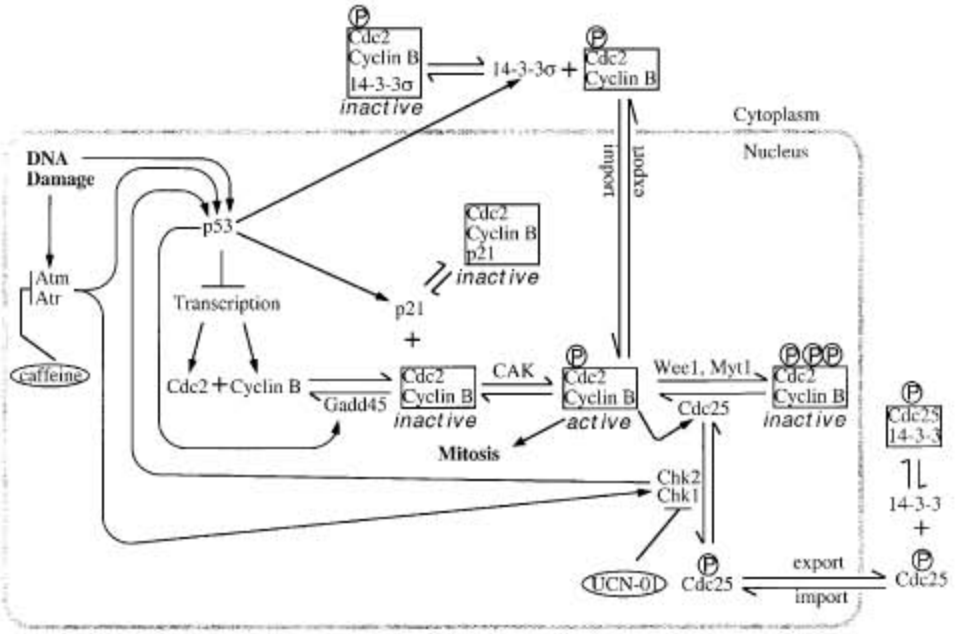

Cdc2
Cyclin B
14-3-3σ
inactive
14-3-3σ +
Cdc2
Cyclin B
Cytoplasm
Nucleus
import
export
DNA
Damage
p53
Cdc2
Cyclin B
p21
inactive
Atm
Atr
Transcription
p21
+
caffeine
Cdc2 + Cyclin B
Gadd45
Cdc2
Cyclin B
inactive
CAK
Cdc2
Cyclin B
active
Wee1, Myt1
Cdc25
Cdc2
Cyclin B
inactive
Mitosis
Chk2
Chk1
UCN-01
Cdc25
export
import
Cdc25
14-3-3
14-3-3
+
Cdc25

Figure 4. Regulation of the Cyclin B/Cdk1 (cdc2)

From (Taylor and Stark 2001). Refer to section 1.4.3 for a detailed description.

1.5 Significance of this work

SIRT1 is an NAD^+-dependent deacetylase, and its gene expression and pharmacological activators can affect the lifespan of multiple organisms. It appears to be crucial for CR-mediated lifespan extension, which is the only regimen that is consistently reported to increase both the mean and maximum lifespan of various organisms, including non-human primates. SIRT1 also influences cell survival, stress resistance, cell differentiation, and glucose and fat metabolism.

Although the importance of SIRT1 in aging research has been established, a couple of important questions have not been addressed, namely, "is SIRT1 regulated in the context of aging and senescence?" and, "is it modified post-translationally?"

To address the first question, I investigated the changes in the level of SIRT1 in aging and senescence, as described in chapter 2 I showed that SIRT1 protein levels decrease with aging both *in vitro* and *in vivo* and correlate with the mitotic activity of cells. This decrease is not due to altered levels of *SIRT1* transcription, suggesting that a post-transcriptional mechanism is involved.

As mentioned in the review of the cell cycle above, each cyclin/Cdk complex functions in specific phases of the cell cycle and Cdk activity is strictly dependent on cyclin levels. After I found that the protein levels of SIRT1 correlate with cell proliferation (see Chapter 2), I investigated the possible regulation of SIRT1 by Cdks. This work is described in Chapter 3. I found that SIRT1 interacts with cyclin B and Cdk1 and is phosphorylated *in vivo*. Similar results were seen *in vitro* and I identified that SIRT1 is phosphorylated by Cyclin B and Cdk1 at Thr530 and Ser540. These

phosphorylation sites and Cyclin/Cdk recognition motifs are conserved among different species. Thus, SIRT1 is a substrate for Cyclin B/Cdk1.

After establishing that SIRT1 is phosphorylated *in vivo* and *in vitro*, I investigated the role of this modification in SIRT1 function. I found that dephosphorylating SIRT1 *in vitro* using phosphatase treatment reduces its NAD^+-dependent deacetylase activity. Furthermore, I investigated the significance of SIRT1 phosphorylation at Thr530 and Ser540 using double mutations for T530A/S540A on SIRT1. ES cells deficient in Sirt1 ($Sirt1^{-/-}$) have a slower growth rate compared to wild-type cells; this phenotype is rescued by wild-type but not AA mutant SIRT1. In addition, SIRT1 rescued the increase in S-phase cells in $Sirt1^{-/-}$ ES cells, while the AA mutant protein increased S-phase fraction in $Sirt1^{+/+}$ ES cells.

Taken together, SIRT1 is phosphorylated by Cyclin B/Cdk1, and this event regulates its deacetylase activity and affects cell proliferation.

Chapter 2: Progressive loss of SIRT1 with cell cycle withdrawal

2.1 SUMMARY

Sir2 is an NAD^+-dependent deacetylase that regulates life-span in yeast, worms and flies. The mammalian orthologs of Sir2 include SIRT1 in humans and mice. In this study, we analyzed the level of SIRT1 in human lung fibroblasts (IMR90) and mouse embryonic fibroblasts (MEFs) from mice with normal, accelerated, and delayed aging. SIRT1 protein, but not mRNA, decreased significantly with serial cell passage in both human and murine cells. Mouse SIRT1 decreased rapidly in prematurely-senescent (p44 Tg) MEFs, remained high in MEFs with delayed-senescence (Igf-1r-/-), and was inversely correlated with senescence-activated beta-galactosidase activity. Reacquisition of mitotic capability following spontaneous immortalization of serially passaged wild-type MEFs restored the level of SIRT1 to that of early passage, highly proliferative MEFs. In mouse and human fibroblasts, I found a significant positive correlation between the levels of SIRT1 and PCNA, a DNA processivity factor expressed during S-phase. In the animal, I found that SIRT1 decreased with age in tissues in which mitotic activity also declines, such as the thymus and testis, but not in tissues such as the brain in which there is little change in mitotic activity throughout life. Again, the decreases in SIRT1 were highly correlated with decreases in PCNA. Finally, loss of SIRT1 with age was accelerated in mice with accelerated aging but was not observed in long-lived GHRKO mice. Thus, as mitotic activity ceases in mouse and human cells in the normal

environment of the animal or in the culture dish, there is a concomitant decline in the level of SIRT1.

2.2 INTRODUCTION

Cellular senescence, which refers to the process by which primary cells in culture lose their ability to divide, has been a useful model for the study of mammalian aging for decades (Hayflick and Moorhead 1961), yet not all or even most of the molecular details by which this process occurs are known. Cellular senescence appears to parallel organismal aging, and cells from mice or humans with various premature aging syndromes undergo senescence in culture more rapidly than do cells from normal mice or humans (Lombard *et al.* 2005). How, or even if, cellular senescence contributes to organismal aging, however, is not known. Senescent cells accumulate *in vivo* as the organism ages, as well as at sites of pathology (Itahana *et al.* 2004). One theory holds that senescence of stem or progenitor cells impairs tissue renewal, limiting the ability of the organism to replace cells that die or wear out with time and leading to the deterioration of the body and frailty we associate with old age.

One molecule that is known to affect senescence is the NAD^+-dependent deacetylase *SIR2* (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000). Although first identified in yeast as a gene involved in mating type switching (Rine and Herskowitz 1987), the *SIR2* gene is highly conserved in organisms ranging from archea to humans (Brachmann *et al.* 1995), where its role is much broader. Over-expression of Sir2 extends replicative life-span in yeast (Kaeberlein *et al.* 1999), and Sir2 orthologs extend

organismal life-span in both worms (Tissenbaum and Guarente 2001) and flies (Rogina and Helfand 2004).

Of the seven Sir2 family homologues (sirtuins) in humans (Frye 1999; Frye 2000), *SIRT1* is most closely related to the *SIR2* gene of *Saccharomyces cerevisiae* (Frye 2000). In contrast to yeast, SIRT1 was found not to extend replicative lifespan when over-expressed in human fibroblasts or epithelial cells under standard culture conditions (Michishita *et al.* 2005), and deletion of the normal allele of *Sirt1* from heterozygous *Sirt1*+/- MEFs in culture dramatically increased replicative life-span (Chua *et al.* 2005). Thus, how SIRT1 is involved in mammalian replicative senescence remains unclear.

The observations from a number of organisms showing that changes in Sir2 expression accompany changes in the ability of cells to divide led us to explore this link in greater detail. I examined the levels of the mammalian Sir2 ortholog, SIRT1, under conditions that alter mitotic activity both *in vitro* and *in vivo*. The results of this study imply that mammalian SIRT1 might interact with the cell cycle on multiple levels.

2.3 RESULTS

2.3.1 The level of SIRT1 decreases with serial cell passage as cells stop dividing

Replicative life-span in yeast cells is sensitive to the dosage of the yeast homologue of *SIRT1*, *SIR2* (Kaeberlein *et al.* 1999). In order to determine if such a relationship exists in mammalian cells, I analyzed the level of SIRT1 in human and murine cells during serial cell passage. Human lung fibroblasts (IMR90) were serially

passaged at a ratio of 1:8 in 10cm plates, and protein levels determined by western blot analysis. As shown in **Fig. 5A**, as the number of population doublings of IMR90 cells increased, there was progressively less SIRT1 in the extracts. Similarly, in normal mouse embryonic fibroblasts (MEFs) passaged at a ratio of 1:4 in 10cm plates, the level of SIRT1 decreased with increasing passage number in MEFs prepared from two different strains of mice (**Fig. 5B**, *ICR* and **Fig. 5C**, *C57Bl6* (B6)).

I next determined if the level of SIRT1 in murine cells in which senescence is accelerated or delayed relative to normal declined at a faster or slower rate, respectively. In MEFs from p44 transgenic mice, which express a short isoform of p53 (p44) and senesce more rapidly than normal MEFs (Maier *et al.* 2004), the rate at which SIRT1 levels declined was also more rapid than in non-transgenic MEFs of the same strain (*ICR*) (**Fig. 5B,** compare *ICR* to *p44+/+*). These observations were confirmed in two independent serial cell passage experiments. Conversely, MEFs that senesce more slowly in culture showed little decline in SIRT1 levels with passage number. *Igf-1r-/-* MEFs, for example, were serially passaged at a ratio of 1:4 until passage 4, then at a ratio of 1:8. These cells did not undergo any of the changes associated with senescence, such as cell flattening and enlargement, even after approximately 34 population doublings (passage 12; data not shown). As shown in **Fig. 5C**, they also showed little if any decline in SIRT1 compared to normal MEFs of the same strain (*C57Bl6*).

To get a rough estimate of how mitotically active the cells were at each successive passage, I determined the level of a DNA polymerase processivity factor, proliferating cell nuclear antigen (PCNA), in the same extracts used for SIRT1 determination. I found

a significant linear correlation between levels of the SIRT1 and PCNA (**Fig. 5A-D**, *F*-test, (**A**): $p<0.05$; (**B**), $p<0.001$; (**C**), $p<0.001$; (**D**), $p<0.01$). I was able to confirm this observation in MEFs that underwent spontaneous immortalization in culture. Following a decline in both SIRT1 and PCNA levels between passage 0 and passage 3, the amounts of both proteins began to increase, reaching pre-senescence levels again at about passage 6 (**Fig. 5D**).

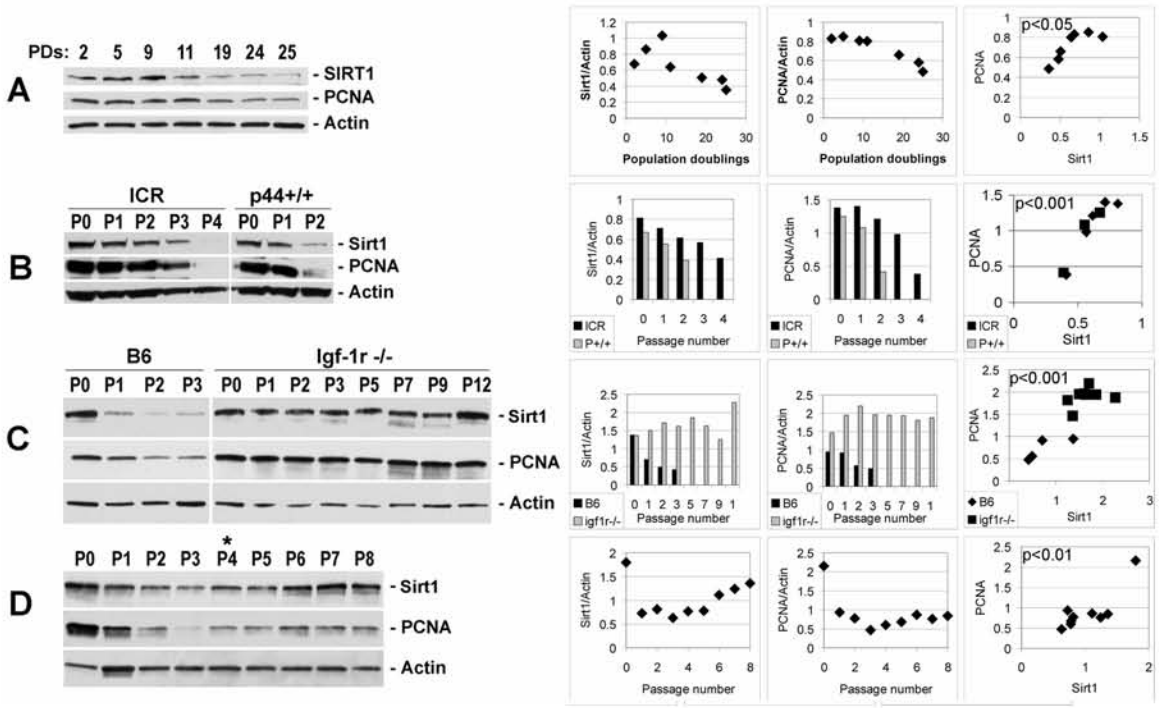

A
PDs: 2 5 9 11 19 24 25
- SIRT1
- PCNA
- Actin
B
ICR
p44+/+
P0 P1 P2 P3 P4 P0 P1 P2
- Sirt1
- PCNA
- Actin
C
B6
Igf-1r -/-
P0 P1 P2 P3 P0 P1 P2 P3 P5 P7 P9 P12
- Sirt1
- PCNA
- Actin
D
P0 P1 P2 P3 * P4 P5 P6 P7 P8
- Sirt1
- PCNA
- Actin
Sirt1/Actin
Population doublings
PCNA/Actin
Population doublings
PCNA
Sirt1
p<0.05
ICR
P+/+
Passage number
p<0.001
B6
igf1r-/-
p<0.01

Figure 5. SIRT1 protein level decreases with serial cell passage and is coupled to PCNA level

(**A**) Western blot of extracts from IMR90 human lung fibroblasts. Note the coordinate decline in SIRT1 and PCNA with serial cell passage.

(**B**) Western blot of extracts from ICR (left) and p44 (right) MEFs. Note that both SIRT1 and PCNA decrease faster in $p44^{+/+}$ MEFs, which senesce prematurely, compared to NT MEFs.

(**C**) Western blots of extracts from B6 (left) and $Igf1r^{-/-}$ (right) MEFs. Note that both SIRT1 and PCNA levels remain high in $Igf1r^{-/-}$ MEFs, which did not senesce in culture, compared to normal MEFs, which did.

(**D**) Western blot of extracts from NT MEFs from ICR strain that underwent spontaneous immortalization at passage 4 (P4*). Note that SIRT1 and PCNA protein levels are coupled to each other and to cellular mitotic activity. Results of densitometric analysis of each blot were plotted and are shown in graphs on the right side of each figure. In each experiment, levels of SIRT1 and PCNA showed significant linear correlation (*F*-test, *p*-values indicated in graphs).

Abbreviations: NT, non-transgenic: P, passage; PDs, population doublings.

2.3.2 SIRT1 is regulated at the protein, not the RNA, level

The observed decline in the level of SIRT1 could have been caused by changes at the transcriptional level or the post-transcriptional level. To distinguish between these possibilities, I analyzed the levels of *SIRT1* mRNA in serially passaged cells. The results are shown in **Fig. 6** for IMR90 cells (**Fig. 6A**), normal *ICR* MEFs (**Fig. 6B**), and p44 transgenic MEFs (**Fig. 6C**). In contrast to protein levels, the level of *SIRT1* mRNA did not decrease significantly with passage [IMR90 cells (**A**): *F*-test, $p>0.1$ for correlation between population doubling and *SIRT1* mRNA level; ICR MEFs (**B**): *t*-test, $p>0.1$ between passage 0 and 3; and, $p44^{+/+}$ transgenic MEFs (**C**): *t*-test, $p>0.05$ between passage 0 and 2]. Furthermore, when I compared protein levels in IMR90 cells (**Fig. 5A**) to mRNA levels (**Fig. 6A**), I did not find a significant linear correlation between the two (**Fig. 6D**: *F*-test, $p>0.1$). Thus, the changes in the level of SIRT1 protein occur post-transcriptionally.

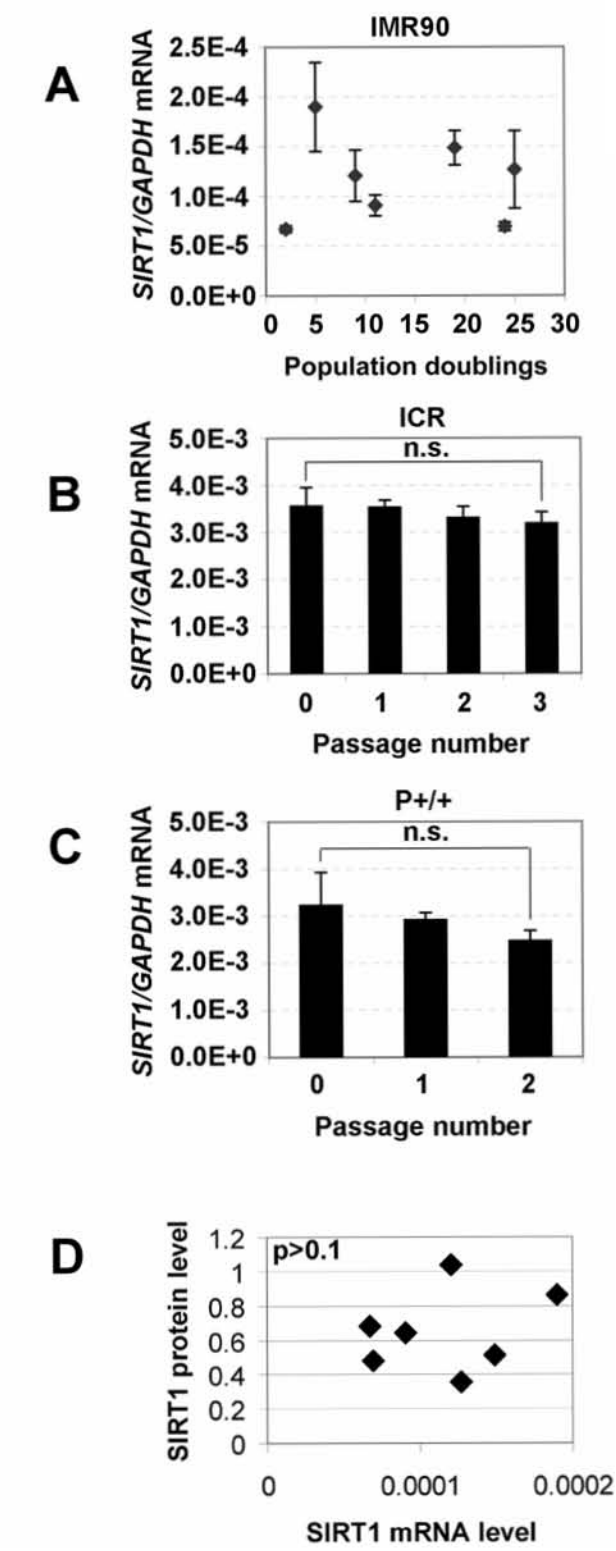

A
IMR90
SIRT1/GAPDH mRNA
2.5E-4
2.0E-4
1.5E-4
1.0E-4
5.0E-5
0.0E+0
0 5 10 15 20 25 30
Population doublings
B
ICR
n.s.
SIRT1/GAPDH mRNA
5.0E-3
4.0E-3
3.0E-3
2.0E-3
1.0E-3
0.0E+0
0 1 2 3
Passage number
C
P+/+
n.s.
SIRT1/GAPDH mRNA
5.0E-3
4.0E-3
3.0E-3
2.0E-3
1.0E-3
0.0E+0
0 1 2
Passage number
D
p>0.1
SIRT1 protein level
1.2
1
0.8
0.6
0.4
0.2
0
0 0.0001 0.0002
SIRT1 mRNA level

Figure 6. *SIRT1* mRNA level does not correlate with number of population doublings *in vitro*

SIRT1 mRNA level was standardized to *GAPDH* mRNA level as determined by qRT-PCR. *SIRT1* mRNA level does not correlate with (**A**) number of population doublings in IMR90 human lung fibroblasts (*F*-test, $p>0.1$), or passage number in (**B**) ICR MEFs (*t*-test, $p>0.1$ between passage 0 and 3) or (**C**) $p44^{+/+}$ transgenic MEFs (*t*-test, $p>0.05$ between passage 0 and 2). Error bars represent the standard deviations of three measurements of each sample. There was no significant linear correlation between levels of SIRT1 mRNA and protein in IMR90 cells (**D**: *F*-test, $p>0.1$)

2.3.3 The level of SIRT1 increases when cells are stimulated to divide

Based on our findings with cells subjected to serial cell passage, I hypothesized that the level of SIRT1 protein in a cell might be linked to its mitotic activity. In order to test this hypothesis, I cultured IMR90 human lung fibroblasts (**Fig. 7A**) and normal *C57Bl6* MEFs (**Fig. 7B**) under different serum concentrations. Both SIRT1 and PCNA increased when cells were stimulated with FBS compared to no stimulation. However, SIRT1 appeared to be more sensitive to serum concentration, reaching maximal induction at a lower concentration than needed for maximal PCNA induction. When cells were treated with aphidicolin, a DNA polymerase inhibitor that should block cells in S-phase, the level of SIRT1 did not go up with serum stimulation (**Fig 7C**). At the same time, the increase in PCNA level indicated that cells had entered S-phase prior to the block.

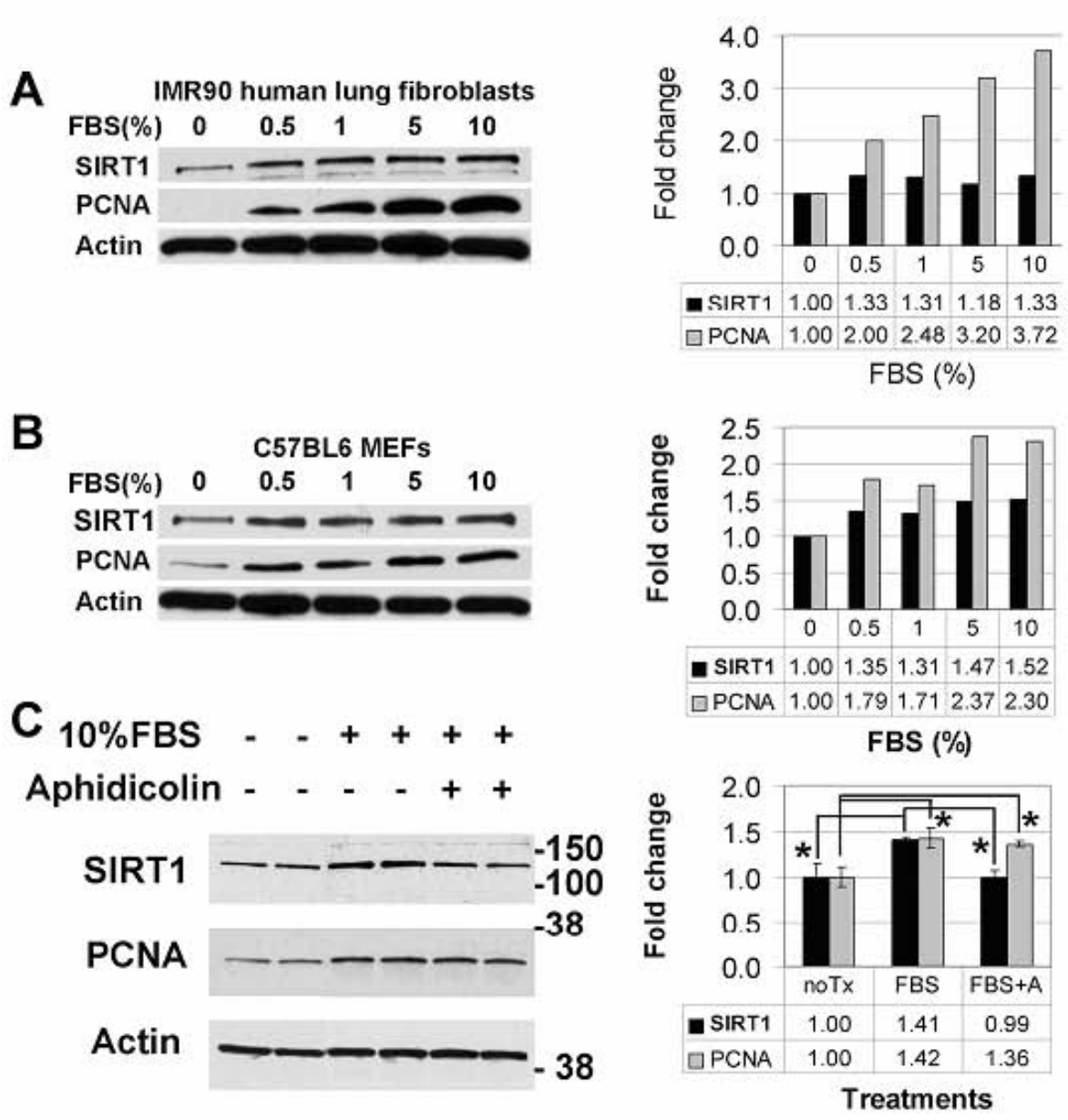

A
IMR90 human lung fibroblasts
FBS(%) 0 0.5 1 5 10
SIRT1
PCNA
Actin
Fold change
4.0
3.0
2.0
1.0
0.0
0 0.5 1 5 10
SIRT1 1.00 1.33 1.31 1.18 1.33
PCNA 1.00 2.00 2.48 3.20 3.72
FBS (%)
B
C57BL6 MEFs
FBS(%) 0 0.5 1 5 10
SIRT1
PCNA
Actin
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
0 0.5 1 5 10
SIRT1 1.00 1.35 1.31 1.47 1.52
PCNA 1.00 1.79 1.71 2.37 2.30
FBS (%)
C
10%FBS - - + + + +
Aphidicolin - - - - + +
SIRT1
PCNA
Actin
-150
-100
-38
- 38
Fold change
2.0
1.5
1.0
0.5
0.0
noTx FBS FBS+A
SIRT1 1.00 1.41 0.99
PCNA 1.00 1.42 1.36
Treatments

Figure 7. SIRT1 and PCNA protein levels are coupled *in vitro*

Western blots of extracts from (**A**) IMR90 human lung fibroblasts and (**B**) C57BL6 MEFs stimulated with 0.5 – 10% FBS. Both SIRT1 and PCNA increased upon serum stimulation in both cell types.

(**C**) Western blot of extracts from non-transgenic ICR MEFs treated for 24 hours with 0.5% serum (no Tx), 10% serum (FBS), or 10% serum with aphidicolin (FBS+A) after 24 hours of serum starvation by 0.5% serum. Asterisks (*) indicates significant difference between two groups (*t*-test, $p<0.05$). In (**C**), anti-SIRT1 monoclonal antibody was used.

2.3.4 SIRT1 decreases specifically in cells that stop dividing

Next, I used histochemical techniques to correlate loss of SIRT1 with the onset of senescence in populations of normal cells undergoing successive cell divisions in culture. Cells with prominent senescence-activated β-galactosidase (SA-βGal) activity (**Fig. 8**, arrowheads, left panel) did not react with an antibody against Sirt1 (middle panel), while cells that showed only slight SA-βGal activity (**Fig. 8**, arrows) retained some Sirt1 reactivity, and cells that showed no SA-βGal activity showed high Sirt1 reactivity. Thus, the decrease in Sirt1 with senescence can be attributed to a decrease in the population of cells that continue to proliferate rather than an overall decrease in Sirt1 expression in all cells.

In summary, these data indicate that the level of SIRT1 in mammalian cells both correlates positively with mitotic activity and decreases with replicative senescence.

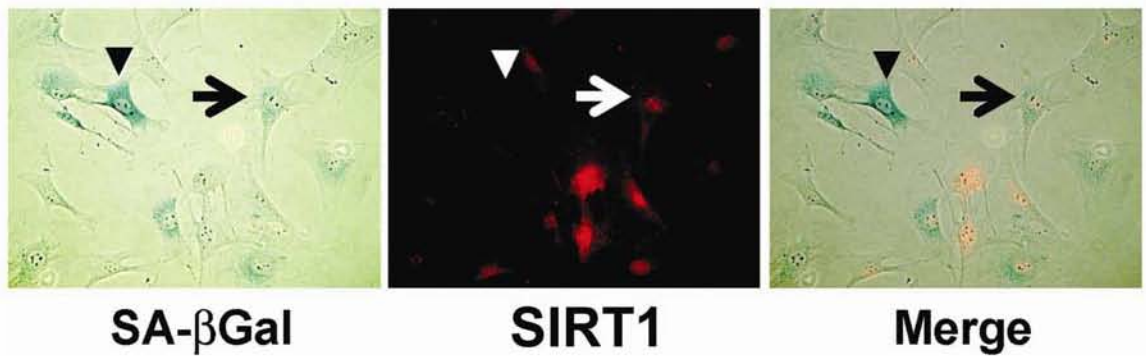

SA-βGal
SIRT1
Merge

Figure 8. Sirt1 is lost specifically in cells with decreased mitotic activity

Sirt1 immunofluorescence (middle) combined with senescence-activated beta-galactosidase (SA-βGal) stain (left). Note that cells with prominent SA-βGal activity (arrowheads) did not react with an antibody against Sirt1, while cells that showed only slight SA-βGal activity retained some Sirt1 reactivity (arrows), and cells that showed no SA-βGal activity showed high Sirt1 reactivity.

2.3.5 SIRT1 and mitotic activity are also coupled in tissues and decline in parallel during aging

In humans and mice, mitotic activity is higher in tissues that have a greater demand for regeneration and cell replacement than in tissues that undergo relatively little cell replacement. The rates of mitotic activity also change as the organism ages. I measured the levels of Sirt1 in an organ that undergoes little cell replacement (brain), and in two organs in which the demand for cell replacement is greater (thymus and testis). I compared the level of Sirt1 in tissues from young adult mice (4 months of age) to those of older mice (12, 18, and 24 months of age) using western blot analysis with antibodies against N- and C-terminal regions of Sirt1. In the brain, which consists mainly of post-mitotic neurons and glial cells that remain capable of proliferation, there was no consistent change in Sirt1 level with age (**Fig. 9A**). The slight increase in PCNA signal may, infact, reflect this glial cell mitotic activity. In all, there was no significant linear correlation between the levels of Sirt1 and PCNA in brain (*F*-test, $p>0.1$). In the testis, where the seminiferous epithelium remains highly mitotically active throughout most of adulthood, the level of Sirt1 was maintained at a relatively constant level, with some decline observed in the oldest animals (**Fig. 9B**). This relatively persistent mitotic activity is reflected in the levels of PCNA, which significantly correlated to the levels of Sirt1 at all ages (**Fig. 9B**: *F*-test, $p<0.001$). In contrast, both Sirt1 and PCNA levels decreased significantly with age in the thymus (**Fig. 9C**). The decline in PCNA reflects the decreased mitotic activity of thymocytes during regression of the thymus, which

occurs in adult animals (Taub and Longo 2005). There was also significant linear correlation between Sirt1 and PCNA levels in thymus (*F*-test, $p<0.001$).

To determine if these changes in Sirt1 protein level in tissues were due to changes in *Sirt1* transcription, I measured the level of *Sirt1* mRNA in the same brain, testis and thymus samples using quantitative real-time RT-PCR. I found no significant correlation between the amount of *Sirt1* transcript and the age of the animal for any of the tissues tested (*F*-test, $p>0.1$). I also did not find any significant linear correlation between the levels of Sirt1 mRNA and protein among tissues examined (*F*-test, $p>0.1$). These results, which are presented in **Fig. 10**, are consistent with similar findings in cultured cells shown in **Fig. 6**.

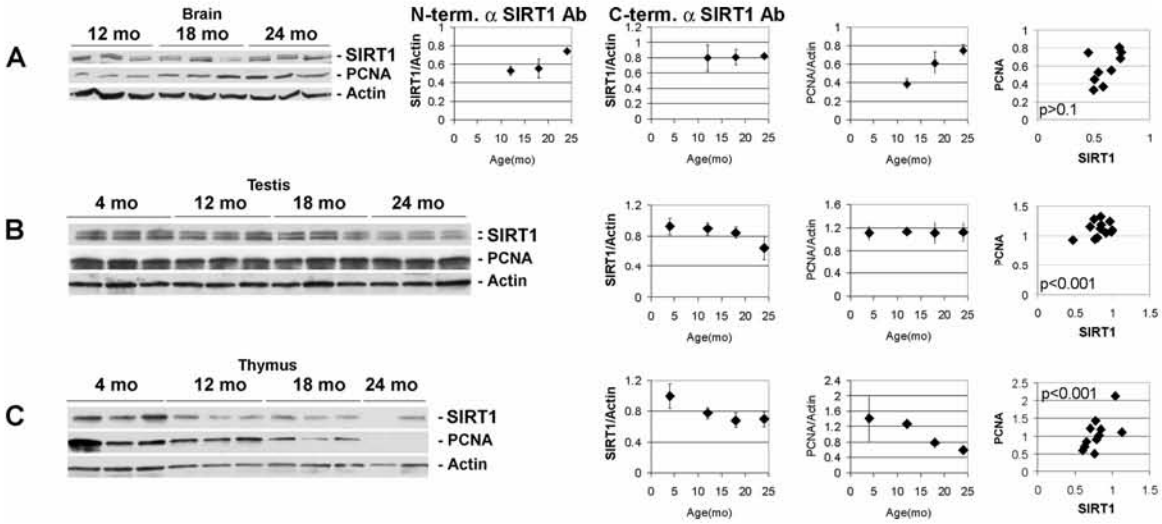

A
Brain
12 mo
18 mo
24 mo
- SIRT1
- PCNA
- Actin
N-term. α SIRT1 Ab
C-term. α SIRT1 Ab
SIRT1/Actin
PCNA/Actin
Age(mo)
PCNA
SIRT1
p>0.1
B
Testis
4 mo
12 mo
18 mo
24 mo
: SIRT1
- PCNA
- Actin
p<0.001
C
Thymus
4 mo
12 mo
18 mo
24 mo
- SIRT1
- PCNA
- Actin
p<0.001

Figure 9. Sirt1 protein level decreases with age in mitotic tissues, but not in post-mitotic tissues

Western blots performed on lysates from B6 male brain (**A**), testis (**B**), and thymus (**C**). Densitometry results for each blot are shown on the right. There was a significant decrease in testis (*F*-test, $p<0.02$) and thymus (*F*-test, $p<0.005$). Brain blot showed an age-dependent increase in Sirt1 with the anti-N-terminal Sirt1 antibody, but not with the anti-C-terminal Sirt1 antibody. Age-dependent changes in Sirt1 in testis and thymus were seen with both anti-N and C-terminal Sirt1 antibodies. Each data point represents an average of the samples from same age, with error bars representing the standard deviations among samples. There were significant linear correlations between levels of Sirt1 and PCNA in testis and thymus (*F*-test, $p<0.001$), but not in brain (*F*-test, $p>0.1$). Abbreviations: mo, month-old.

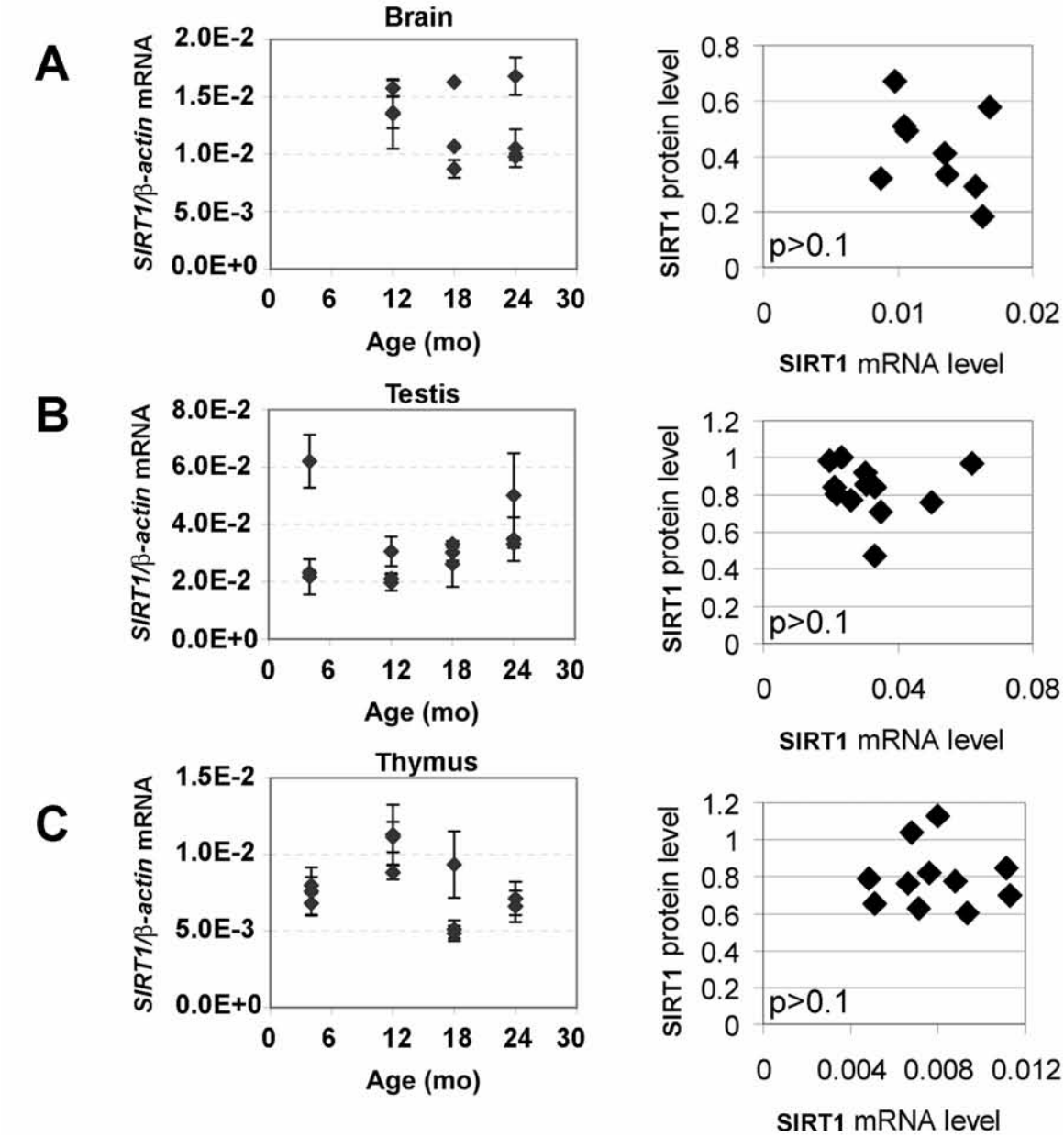

A
Brain
SIRT1/β-actin mRNA
2.0E-2
1.5E-2
1.0E-2
5.0E-3
0.0E+0
0
6
12
18
24
30
Age (mo)
SIRT1 protein level
0.8
0.6
0.4
0.2
0
p>0.1
0
0.01
0.02
SIRT1 mRNA level
B
Testis
SIRT1/β-actin mRNA
8.0E-2
6.0E-2
4.0E-2
2.0E-2
0.0E+0
0
6
12
18
24
30
Age (mo)
SIRT1 protein level
1.2
1
0.8
0.6
0.4
0.2
0
p>0.1
0
0.04
0.08
SIRT1 mRNA level
C
Thymus
SIRT1/β-actin mRNA
1.5E-2
1.0E-2
5.0E-3
0.0E+0
0
6
12
18
24
30
Age (mo)
SIRT1 protein level
1.2
1
0.8
0.6
0.4
0.2
0
p>0.1
0
0.004
0.008
0.012
SIRT1 mRNA level

Figure 10. *Sirt1* mRNA level does not correlate with age *in vivo*

qRT-PCR determination of *Sirt1* mRNA levels in brain (**A**), testis (**B**), and thymus (**C**). No significant correlation with *Sirt1* mRNA level and age ($p>0.1$ by *F*-test for brain, testis and thymus). *Sirt1* mRNA level was standardized to *beta-actin* mRNA levels for each sample. The same tissues were used for these qRT-PCR data as were used for western blots in **Fig.5A-C**. Error bars represent the standard deviations of three measurements of each sample. There was no significant linear correlation between levels of Sirt1 mRNA and proteins in tissues examined (*F*-test, $p>0.1$).

2.3.6 Accelerated or delayed aging alters the rate of Sirt1 decline

As a final test of the link between the level of SIRT1 and mitotic activity, I utilized two mouse models of aging, the p44 transgenic mouse, which exhibits accelerated aging, and the growth hormone-receptor knock-out (GHRKO) mouse, in which aging is delayed. In the accelerated aging model, I found that Sirt1 in the testis of p44 transgenic mice decreased to a greater extent and in younger animals than in non-transgenic mice of the same strain (*ICR*) (**Fig. 11A**). This is consistent with the premature reproductive senescence and decreased spermatogenesis seen in these mice (Maier *et al.* 2004). Sirt1 levels in the thymus of p44 transgenic mice also decreased at younger ages than in NT mice (**Fig. 11C**).

In the GHRKO mice, on the other hand, and in their normal littermates, I found that Sirt1 and PCNA levels were maintained in the testes with age (**Fig. 11B**). In the thymus, however, Sirt1 levels declined with age in both GHRKO mice and normal littermates, and there was no difference in the rate of Sirt1 decline between two groups (**Fig. 11D**). Significant linear correlations between the levels of Sirt1 and PCNA were observed in the thymus (*F*-test, $p<0.05$ in **Fig. 11C**, $p<0.005$ for **Fig. 11D**). There was no significant linear correlation between the levels of Sirt1 and PCNA in testis.

These data with tissues from normally aging mice and from mutant mice in which the rate of aging is disturbed reinforce the observation that the loss of Sirt1 is directly proportional to the loss of mitotic activity, and are the *in vivo* equivalent of data obtained using cells undergoing replicative senescence in culture.

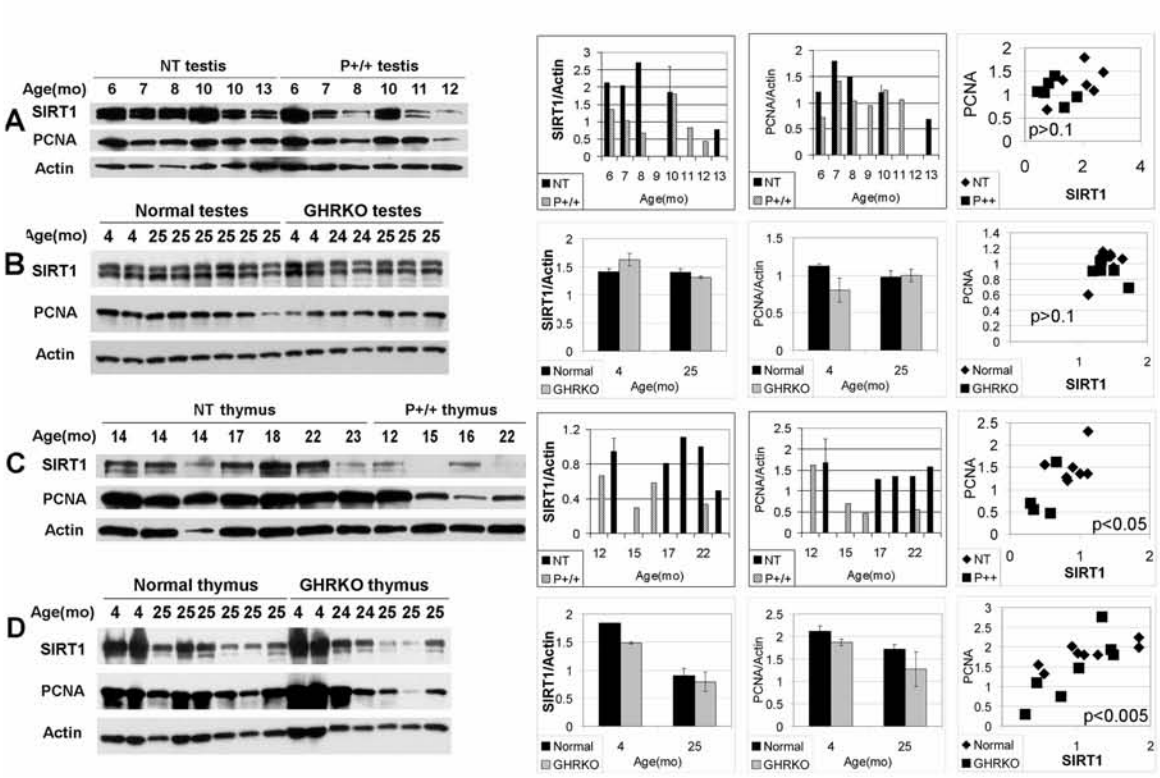

A
NT testis
P+/+ testis
Age(mo) 6 7 8 10 10 13 6 7 8 10 11 12
SIRT1
PCNA
Actin
B
Normal testes
GHRKO testes
Age(mo) 4 4 25 25 25 25 25 25 4 4 24 24 25 25 25
SIRT1
PCNA
Actin
C
NT thymus
P+/+ thymus
Age(mo) 14 14 14 17 18 22 23 12 15 16 22
SIRT1
PCNA
Actin
D
Normal thymus
GHRKO thymus
Age(mo) 4 4 25 25 25 25 25 25 4 4 24 24 25 25 25
SIRT1
PCNA
Actin
SIRT1/Actin
PCNA/Actin
Age(mo)
NT
P+/+
Normal
GHRKO
PCNA
SIRT1
p>0.1
p<0.05
p<0.005
P++

Figure 11. The rate at which Sirt1 protein level declines *in vivo* is affected by the rate of aging

(**A**) Western blot of extracts from NT and $p44^{+/+}$ testis. Sirt1 protein level decreases faster in prematurely-aging $p44^{+/+}$ testis, than in normal (NT) testis.

(**B**) Western blot of GHRKO and normal male testis. There was no major decline in Sirt1 and PCNA levels in either genotype.

(**C**) Western blots of Sirt1 level in NT and $p44^{+/+}$ thymus.

(**D**) Western blot of extracts from Normal and GHRKO thymus. Sirt1 level declined both in GHRKO thymus and normal counterparts, but there was no difference in the rate of decline. Numbers above the lanes of each western blot indicate the age (month) of animal analyzed. Results of densitometric analysis of each blot are shown on the right. Significant linear correlations between levels of Sirt1 and PCNA were observed in (**C**) and (**D**), but not in (**A**) and (**B**) (*F*-test). 25mo group for GHRKO tissues include both 24 and 25mo samples.

Abbreviation: GHRKO, growth hormone receptor-knockout; mo, month old; NT, non-transgenic; P+/+, p44 +/+ transgenic mice.

2.4 DISCUSSION

In this study, I have explored the relationship between changes in the level of the mammalian homologues of Sir2, SIRT1 and Sirt1, and changes in mitotic activities of cells undergoing senescence in culture or as a consequence of the aging process in the animal. When I began this study, I thought that the most likely effect of SIRT1 would be to facilitate mitosis via a pathway upstream of cell cycle entry. This assumption was based largely on the effects of calorie restriction or nutrient withdrawal on the level of SIRT1 expression (Cohen *et al.* 2004; Nemoto *et al.* 2004; Rodgers *et al.* 2005), and on the effect of SIRT1 on cell survival under condition that evoke a p53 response (Luo *et al.* 2001; Vaziri *et al.* 2001; Langley *et al.* 2002). Results of our study imply, however, that the effects of SIRT1 on life-span may lie downstream of the cell cycle and be coupled to the cellular machinery of mitosis itself. The key distinction lies in the fact that nutrient withdrawal can affect the level of *SIRT1* transcription (Nemoto *et al.* 2004), whereas mitosis appears to affect the level of SIRT1 protein post-transcriptionally. I found no evidence for fluctuations in the amount of *SIRT1* mRNA under conditions that altered mitotic activity both *in vitro* and *in vivo* in any of the cells or tissues I analyzed. What I did find was that replicative cellular senescence in human and mouse fibroblasts was accompanied by a decrease in the level of SIRT1 protein. Other studies with fibroblasts (Michishita *et al.* 2005), human lung and breast cancer cells (Ota *et al.* 2006), and primary neuroblasts (Horio *et al.* 2003) have reported similar links between low levels of SIRT1 and reduced cellular proliferation. In addition, cells from mice deficient in Sirt1 show reduced proliferative capability, a finding which is consistent with the reduced

tissue volume seen in tissues such as the pituitary in these mice (Lemieux *et al.* 2005). I found, furthermore, that the level of SIRT1 varies directly with the level of PCNA, which is expressed during DNA replication (Paunesku *et al.* 2001). In cells that senesce prematurely (p44+/+ MEFs), coordinate decreases in Sirt1 and PCNA occurred at earlier cell passages than in normally senescing cells. Igf-1r-null cells grow more slowly and undergo crisis *in vitro* later than their wild-type counterparts (Sell *et al.* 1994). Loss of Igf-1r in mice confers resistance to oxidative stress (Holzenberger *et al.* 2003), which is known to play a major role in murine cell senescence *in vitro* (Parrinello *et al.* 2003). In Igf-1r-/- MEFs, these decreases in Sirt1 and PCNA were not observed while cells remained mitotically active and lacked the morphological changes typical of senescent cells. When wild-type MEFs underwent spontaneous immortalization and re-entered the cell cycle, Sirt1 and PCNA levels went up again concomitant with increased mitotic activity. Finally, Sirt1 immunofluorescence inversely correlated with senescence-activated beta-galactosidase activity, a marker for senescence. Thus, in both human and mouse fibroblasts, the level of SIRT1 is linked to the activity of the cell cycle.

I confirmed these results by experimentally manipulating cell cycle activity with serum and a cell cycle inhibitor, aphidicolin. I found that SIRT1 level was higher in fibroblasts that were stimulated with serum, and that PCNA level changed accordingly. I found that the level of SIRT1 was very sensitive to serum stimulation, reaching maximal levels at low concentration (**Figs. 7A&B**), and that the increase could be blocked by aphidicolin (**Fig. 7C**). These data indicate that SIRT1 increases when cells are allowed to go through mitosis and suggest that cell cycle activity might stabilize the protein after S-

phase. In fact, I detect two SIRT1 bands at about 110kD in murine tissues and cells (see **Figs. 5C&D, 7C, 9A, 9B, 11**) as well as in human cells (see **Fig. 7A**), which could be indicative of two different post-translationally modified forms of the protein. These two bands are detected by both anti-N-terminal and anti-C-terminal SIRT1 polyclonal antibodies and an anti-SIRT1 monoclonal antibody. Post-translational modifications such as phosphorylation and ubiquitination are known to affect protein migration in gels.

The organization of DNA into chromatin and chromosomal structures plays a central role in many aspects of cell biology in eukaryotes. DNA, along with chromatin and other modifications, must all be replicated during mitosis (Ehrenhofer-Murray 2004). Because SIRT1 deacetylates histones (Imai *et al.* 2000), the demand for SIRT1 may go up in proliferating cells. Then, as cells stop dividing, the demand for SIRT1 would decrease and the level of the protein decline accordingly.

Replicative senescence of human and mouse cells in culture, although similar in phenotype, is thought to occur primarily by different mechanisms. In human cells senescence is linked to telomere attrition, while in mouse cells, it is linked to sub-lethal oxidative damage suffered under standard culture conditions (Parrinello *et al.* 2003; Busuttil *et al.* 2004; Shay and Wright 2005). Normal cells senesce following any one of a number of stressors, such as DNA damage, damage to chromatin structure, and oxidation (Serrano and Blasco 2001; Lloyd 2002). All of these ultimately affect the ability of a cell to undergo mitosis. In this study, I found that conditions that alter mitotic activity in fibroblasts control the level of SIRT1. I also found that the levels of Sirt1 and mitosis were linked in tissues from mice undergoing normal and accelerated aging. Sirt1

decreased with age only in tissues in which mitotic activity also slowed. The only exception to this can be explained by the fact that aging is segmental in both animal models used in these studies. For example, in the thymus, Sirt1 levels declined with age in both GHRKO mice and normal littermates, and there was no difference in the rate of Sirt1 decline between two groups (**Fig. 11D**). I conclude that this aspect of aging may not be delayed in GHRKO mice. GHRKO mice also have low serum IGF-1, decreased length and diameter of seminiferous tubules, decreased testosterone secretion upon luteinizing hormone treatment, and decreased fertility (Chandrashekar *et al.* 2004). These endocrinological changes can account for the loss of linear correlation between Sirt1 and PCNA in GHRKO testis (**Fig. 11B**). Finally, the testes of p44 transgenic mice undergo degeneration accompanied by abnormal Leydig cell proliferation seen in this tissue (**Fig. 11A**) (Maier *et al.*, manuscript submitted). This could account for the loss of linear correlation between Sirt1 and PCNA.

In Summary, as mitotic activity ceases in mouse and human cells in the normal environment of the animal or in the culture dish, there is a concomitant decline in the level of SIRT1. These results strongly imply that the level of SIRT1 and replicative senescence may be influenced by a common mechanism, such as the machinery that drives mitosis.

2.5 Experimental procedures

2.5.1 Cell culture

MEFs were separated from s.c. tissue of embryos at 13.5 postcoital days, grown in DMEM with 10% fetal bovine serum and high glucose, unless otherwise noted. IMR90 human lung fibroblasts were obtained from ATCC. Cells were cultured in 37°C with 5% CO_2, with DMEM with high glucose (GIBCO#11885) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin (Invitrogen). For the serum comparison experiment, C57BL6 MEFs and IMR90 fibroblasts were plated subconfluently in 6 well plates in DMEM with 10% FBS, then starved in 0.5% FBS overnight, followed by incubation with 0%, 0.5%, 1%, 5%, or 10% FBS for 48 hours. For the growth factor treatment experiment, non-transgenic MEFs from ICR strain were starved with 0.5% FBS for 24 hours, followed by 0.5% FBS, 10% FBS, or 10% FBS with aphidicolin (4ug/ml, EMD Biosciences) for 24 hours.

2.5.2 Serial cell passage

IMR90 human lung fibroblasts were serially passaged at a ratio of 1:8 in 10cm plates. Mouse embryonic fibroblasts (MEFs) from wild-type ICR strain and $p44^{+/+}$ transgenic mice were serially passaged at a ratio of 1:4 in 10cm plates. MEFs from wild type C57BL6 strain was serially passaged at a ratio of 1:4, whereas MEFs from $Igf\text{-}1r^{-/-}$ mice were serially passaged at a ratio of 1:4 till passage 4, then at a ratio of 1:8 from passage 5. ICR MEFs that underwent spontaneous immortalization were serially

passaged at a ratio of 1:8. The number of cells in each plate was measured and used to calculate estimated population doublings at each passage in IMR90 cells.

2.5.3 Protein isolation

Tissue samples were first pulverized in liquid nitrogen. Cells in culture were first washed with PBS. Cell lysis buffer (1/10 RIPA buffer, supplemented with 50mM Tris, 137mM NaCl, 10mM NaF, 1mM EDTA, 0.2% sarkosyl, 1mM DTT and 10% glycerol) in the presence of inhibitors (phosphatase inhibitor cocktails (Sigma), Protease inhibitor cocktail (Sigma), Trichostatin A, and Nicotinamide) unless otherwise mentioned, were applied to pulverized tissue or cells *in vitro*, then incubated on ice for 30 minutes. DNA was sheared using 25G needle syringe. Samples were centrifuged at maximum speed for 10 minutes in 4°C to remove any insoluble material.

2.5.4 Western blot analysis

Western blots were performed using Biorad western blot kit (Biorad). Protein from each samples (20-30ug) were loaded on 4-20% gel or 10% gel, and proteins separated by molecular weight. After transfer to Protran nitrocellulose membrane (Perkin-Elmer), the membranes were blocked in 5% milk/0.05% Tween-20 in PBS for 1hr, then hybridized with primary antibody overnight in 4°C. After washing with PBS-T/milk for 5 minutes three times, secondary antibodies were applied and incubated for 1hr. After another 3 washes with PBS-T/milk for 10 minutes each, either Pico or Femto chemilluminescence substrates (Pierce) were used to develop western blots and X-ray

films exposed accordingly. The following antibodies were used: Peroxidase-conjugated AffiniPure Goat-anti-Mouse IgG (Jackson ImmunoResearch #115-035-100, 1: 10,000), Peroxidase-conjugated AffiniPure Goat anti-Rabbit IgG (Jackson ImmunoResearch #110-035-144, 1:10,000), anti-Actin monoclonal antibody (MP Biomedicals #69100, 1: 1,000,000), anti-PCNA polyclonal antibody (Santa Cruz Biotechnology #sc-7907, 1: 1,000), anti-SIRT1 N-terminal polyclonal antibody (Upstate Biotech #07-131, 1: 1,000), anti-SIRT1 C-terminal polyclonal antibody AS-16 (Sigma #S5313, 1: 1,000), and anti-SIRT1 monoclonal antibody 2G1/F7 (Upstate Biotech #05-707, 1: 1,000). For SIRT1 western blots, anti-SIRT1 N-terminal polyclonal antibody was used unless otherwise mentioned.

2.5.5 Western blot densitometry

Films were scanned with Molecular Dynamics Densitometer (GE) and densitometry was performed using ImageQuant5.0 (GE). SIRT1, PCNA, and Actin signals of each sample were measured and SIRT1 and PCNA levels of each sample were standardized to the levels of Actin.

2.5.6 RNA and cDNA preparation

Total RNA from tissues was harvested with 1ml of TriReagent (Molecular Research) after tissues were pulverized in liquid nitrogen. After isolation of RNA, total RNA was treated with RNase-free DNase I (Roche) for 1hr, and RNeasy (Qiagen) was used to purify RNA. Purity and amount of RNA were determined spectrophotometrically

(Eppendorf). Total RNA from cells was harvested in a similar fashion except 0.5ml of TriReagent was used. Reverse transcription reactions to generate cDNAs were performed by SuperScript III (Invitrogen) using 1ug of total RNA as a template and random hexamer as primers. Reactions were performed at 50°C for 1 hour for tissues and for 3 hours for cells *in vitro*.

2.5.7 Quantitative real-time RT-PCR

RT-PCR was performed in two steps. First cDNA was prepared as described above, then 1ul of cDNA was used to perform real-time PCR. DNA FastStart SybrGreenI kit (Roche) and LightCycler (Roche) was used for real-time PCR. The specificity of the reaction was assessed by the melting curve analysis. Each cDNA sample was measured three times. The following primers were used.

Murine *Sirt1* primers:

forward (1918-1933): GTAAGCGGCTTGAGGG

reverse (2132-2147): TTCGGGCCTCTCCGTA

Human *SIRT1* primers:

forward (428-453): GAGATAACCTTCTGTTCGGTGATGAA

reverse (595-621): CGGCAATAAATCTTTAAGAATTGTTCG

Beta-actin primers:

forward (412-431): CCCTAAGGCCAACCGTGAAA

reverse (500-520): ACGACCAGAGGCATACAGGGA

GAPDH primers:

forward (529-548): TGCACCACCAACTGCTTAGC

reverse (595-615): GGCATGGACTGTGGTCATGAG

2.5.8 Statistical analysis

Following densitometry, signal intensities were normalized to loading controls (actin) and *F*-tests were performed to check for significant correlation between age of tissues and SIRT1 signals, levels of SIRT1 and PCNA, and levels of SIRT1 mRNA and protein using Microsoft Excel. For qRT-PCR of tissues, *F*-tests were applied after *SIRT1* transcript levels were adjusted to those of *beta-actin*. For qRT-PCR of cells *in vitro*, after *SIRT1* transcript levels were adjusted to *GAPDH* transcript levels, *F*-test was applied for IMR90 data. For ICR and $p44^{+/+}$ data and data from cell cycle manipulation with aphidicolin, *t*-tests were performed using Microsoft Excel.

2.5.9 Senescence-activated beta-galactosidase staining followed by Sirt1 immunofluorescence

Wild-type MEFs from ICR strain which were passed 3 times at a ratio of 1:4 were washed in PBS once and fixed with 0.5% glutaraldehyde in PBS for 5 minutes, then washed twice in PBS (pH 7.2) with 1mM $MgCl_2$. Cells were stained in X-Gal solution consisting of PBS at pH6.0 containing 1mg of X-gal (5-bromo-4-chloro-3-indolyl—D-galactopyranoside), 0.5mM potassium ferrocyanide, 0.5mM potassium ferricyanide, 150mM NaCl, 1mM $MgCl_2$ overnight at 37°C. The following day, cells were rinsed in PBS once, demasked with ice-cold 0.1N HCl for 15 minutes followed by incubation in

ddH_2O for 5 minutes twice, in PBS containing 5mM $MgCl_2$ and 0.1% Triton-X-100 for 5 minutes, in ddH_2O for 5 minutes twice and in PBS for 2 minutes. Sections were blocked with PBS containing 10% normal goat serum, 3% bovine serum albumin and 0.1% Triton-X-100 for 30 minutes, followed by incubation with anti-Sir2 antibody (Upstate #07-131, 1: 200) overnight. Cells were incubated for 2 hours with Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probe #A11036, 1:300) as a secondary antibody. After washing cells with PBS, cells were mounted with Fluormount G (Southern Biotech #0100-01).

2.6 Acknowledgements

I thank Zak Kohutek for preliminary experiments, Dr. Renato Baserga (Thomas Jefferson University) for Igf-1r -/- MEFs, and Dr. Jeffrey Corwin (UVA) for the use of the Roche LightCycler and Eppendorf spectrophotometer. I thank Marty Wilson (SIU) and Wendy Gluba (UVA) for coordinating tissue preparation, and NIA tissue bank for providing us with aging *C57BL6* mice tissues. I thank Biomolecular Research Facility at UVA Cancer Center for their services.

This work was supported by PHS grant NIH AG20915 (H.S.), NIH AG198899 (A.B.), a pilot grant from the Institute on Aging at the University of Virginia (B.M.) and a pilot grant from the Carilion Institute for Collaborative Research between Virginia Polytechnical Institute and the University of Virginia (B.M.). H.S. is a fellow of the Ellison Medical Foundation.

Chapter 3: SIRT1 is phosphorylated by Cyclin B/Cdk1 and SIRT1 phosphorylation modulates its deacetylase activity and cell proliferation

3.1 SUMMARY

SIRT1 is an NAD^+-dependent deacetylase and the mammalian homolog of yeast Sir2. Increased Sir2 activity confers survival advantage in yeast, worms, flies, and mammalian cells. Resveratrol, a pharmacological activator of SIRT1, improves the life span and health of mice on a high-calorie diet. Although other classes of histone deacetylases have been reported to be modulated by phosphorylation, post-translational modifications of SIRT1 have not been described in depth. I reported previously that the level of SIRT1 protein is regulated by the level of mitotic activity in cells (Sasaki *et al.* 2006), which is regulated in turn by the activities of cyclin-dependent kinases (Cdks). Thus, I tested the hypothesis that SIRT1 is phosphorylated by Cdks.

I found that SIRT1 is phosphorylated at 13 residues *in vivo*. Some of these phosphorylated residues, as well as cyclin recognition motifs and minimal- and full-Cdk substrate motifs, are conserved among SIRT1 orthologs of different species. SIRT1 forms a complex with cyclin B1 and Cdk1 *in vivo*, and is phosphorylated by cyclin B/Cdk1 at threonine 530 and serine 540 *in vitro*. To address the significance of SIRT1 phosphorylation, SIRT1 was dephosphorylated *in vitro* by phosphatases, resulting in decreased NAD^+-dependent deacetylase activity. Furthermore, SIRT1 with T530A/S540A double mutation (AA) did not rescue the slower cell proliferation in $Sirt1^-$

$^{/-}$ ES cells and mouse embryonic fibroblasts, or the increase in the fraction of S-phase cells in Sirt1$^{-/-}$ ES cells. I conclude that SIRT1 is a substrate for cyclin B/Cdk1, and the phosphorylation of SIRT1 modulates its deacetylase activity and affects cell proliferation.

3.2 INTRODUCTION

SIR2 is an NAD^+-dependent deacetylase (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000). Although first identified in yeast as a gene involved in mating type switching (Rine and Herskowitz 1987), the *SIR2* gene is highly conserved in organisms ranging from archea to humans (Brachmann *et al.* 1995). Of the seven Sir2 family homologues (sirtuins) in humans (Frye 1999; Frye 2000), *SIRT1* is most closely related to the *SIR2* gene of *Saccharomyces cerevisiae* (Frye 2000). Over-expression of Sir2 extends replicative life-span in yeast (Kaeberlein *et al.* 1999), and Sir2 orthologs extend organismal life-span in both worms and flies (Tissenbaum and Guarente 2001; Rogina and Helfand 2004). Recently, it was shown that resveratrol, a pharmacological activator of SIRT1, can improve the life span and health of mice on a typical "western" (high-calorie) diet (Baur *et al.* 2006; Lagouge *et al.* 2006).

I previously reported that the level of SIRT1 is coupled to the level of mitotic activity in cells both *in vitro* and *in vivo* (Sasaki *et al.* 2006). Changes in SIRT1 protein level were not associated with changes in mRNA level, suggesting that SIRT1 could be regulated post-transcriptionally. However, other than phosphorylation of serine 27 and serine 47 on SIRT1, which were detected in general screens of nuclear phospho-proteins

in HeLa cell extracts by mass spectrometry (Beausoleil *et al.* 2004), post-translational modifications of this important protein have not been reported.

The cell cycle is regulated by various combinations of cyclin and cyclin-dependent kinases (Cdks), and each cyclin/Cdk complex functions during a specific phase of the cell cycle. In somatic cells, cyclin D/Cdk 4, 6 is active during the movement through G_1 phase and into S phase. Cyclin E/Cdk 2 complex becomes active at late G_1 phase into S phase. Cyclin A/Cdk2 becomes active during S phase. The Cyclin B/Cdk1 complex is activated upon passing the G_2/M checkpoint and inactivated upon entry into anaphase (Nigg 2001).

In this work, I investigated potential mechanisms by which SIRT1 might be regulated by the cell cycle. I hypothesized that SIRT1 might be modulated by phosphorylation conferred by these cyclin-dependent kinases. I also explored the hypothesis that phosphorylation might regulate the deacetylase activity of SIRT1, as it is known to do with other classes of protein deacetylases, such as HDAC1 and HDAC2 (Pflum *et al.* 2001; de Ruijter *et al.* 2003). As described below, I found that SIRT1 is phosphorylated by cyclin B/Cdk1, and that phosphorylation regulates its deacetylase activity and affects cell proliferation.

3.3 RESULTS

3.3.1 SIRT1 is phosphorylated at 13 residues *in vivo*

To determine if SIRT1 is a phosphoprotein, I stained gels containing affinity-purified FLAG-SIRT1 separated by SDS-PAGE with Pro-Q Diamond phosphoprotein reagent. I also performed western analysis using an antibody that detects the phosphorylated serine residue in the consensus Cdk recognition motif (K/R-S*-P-x-K/R). As shown in **Fig. 12a**, both the anti-phospho Serine Cdk antibody and the ProQ reagent detect a protein that migrates to the same position in the gel as FLAG-SIRT1 (120kD; lane marked "-"). The signals decreased in a dose-dependent manner following treatment with lambda protein phosphatase (λppase). Although reaction with the anti-Cdk phospho-serine antibody was lost at a low dose of λppase, some reactivity with the phosphoprotein stain, which detects all phospho-residues, was still visible even after treatment with high doses of λppase and required overnight treatment for complete removal. This difference in sensitivity to λppase treatment of the two detection methods implied that SIRT1 could be phosphorylated at multiple residues, and could contain phospho-threonine and phospho-tyrosine residues in addition to phospho-serine.

To identify the residues of SIRT1 that are phosphorylated *in vivo*, I analyzed affinity-purified FLAG-SIRT1 by mass spectrometry. The analysis revealed 13 phosphorylation sites (**Table I, Fig. 12b**), including threonine 530 and serine 540. Phosphorylation was restricted to serine and threonine residues; there were no phosphorylated tyrosine residues in the samples examined. All the phosphorylation sites

identified are located in either the N-terminal domain or the C-terminal domain of SIRT1, and not in the conserved core catalytic domain (Chang *et al.* 2002; Avalos *et al.* 2004). I confirmed the preliminary finding that Ser27 and Ser47 are phosphorylated, as previously reported following analysis of HeLa cell nuclear extracts by phosphoprotein mass spectrometry (Beausoleil *et al.* 2004).

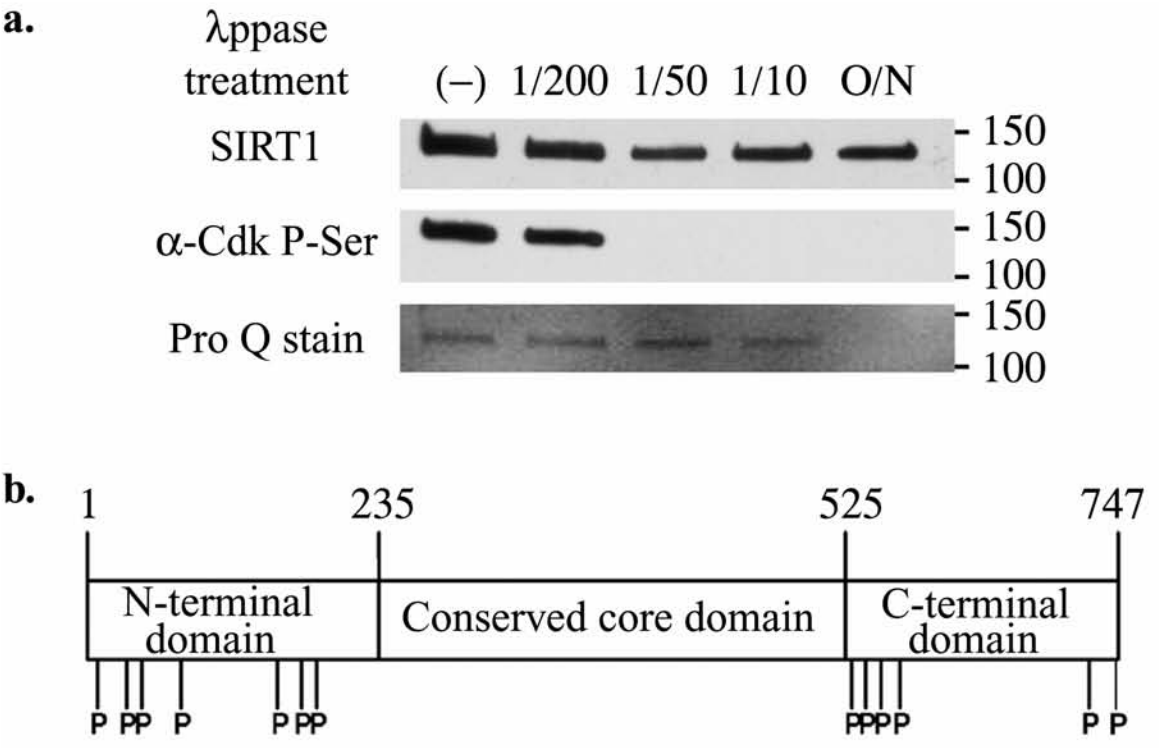

a.
λppase
treatment
(–) 1/200 1/50 1/10 O/N
SIRT1
α-Cdk P-Ser
Pro Q stain
150
100
150
100
150
100
b.
1
235
525
747
N-terminal domain
Conserved core domain
C-terminal domain
P P P P P P P P P P P P P P

Figure 12. SIRT1 is a phosphoprotein

a. Affinity-purified SIRT1 is phosphorylated, and can be dephosphorylated by λppase. Top panel, western blot of total SIRT1; middle panel, western blot of SIRT1 phosphorylated at the Cdk consensus site; bottom panel, ProQ-diamond stained blot showing total phosphorylated SIRT1. Numbers above the lanes indicate the relative doses of λppase used to treat FLAG-SIRT1 for 1 hour. Overnight treatment (O/N) was done with a 1/10 dilution of λppase.

b. Schematic of the phosphorylated residues in SIRT1 identified by mass spectrometry, as shown in **Table I**.

P, phosphorylated residue.

Table I: Phospho-peptide mapping of FLAG-SIRT1 by mass spectrometry

Sequence of Identified Peptides	Sites
MADEAALALQPGGS*PSAAGADR	S14
EAAS*SPAGEPLR	S26
EAASS*PAGEPLR	S27
MADEAALALQPGGS*PSAAGADREAAS?S?PAGEPLR	S14, and S26 or S27
S*PGEPGGAAPER	S47
DNLLFGDEIITNGFHS?CES?DEEDR	S159 or S162
ASHASS*SDWTPRPR	S173
ASHAS*S*SDWTPRPR	S172 and S173 likely (2 out of 3 sites within S172-S174)
ELAYLSELPPT*PLHVSEDSSSPER	T530
ELAYLSELPPTPLHVSEDS?S?S?PER	1 of 3 sites within S538-S540
ELAYLSELPPTPLHVS?EDS?S?S?PER	2 of 4 sites within S535 and S538-S540
T?S?PPDSSVIVTLLDQAAK	T544 or S545
AGGAGFGT*DGDDQEAINEAISVK	T719
QEVTDMNYPSNKS*	S747

Phosphorylation is indicated by an asterisk (*). A site that could not be ruled out is indicated by a question mark (?). Two independent samples of affinity-purified FLAG-SIRT1 were analyzed. The results were consistent between the two, except for S747 phosphorylation, which was detected in only one of the samples.

3.3.2 Cyclin recognition motifs and Cdk substrate residues are conserved among orthologs of SIRT1

To determine if the phosphorylation sites identified by mass spectrometry were evolutionarily conserved, I compared the amino acid sequences surrounding each phosphorylated residue in 12 different species (**Table II**). Among the phosphorylated residues listed in **Table I**, Ser159, Ser162, Ser172, Ser173, Thr530, Ser535, Ser538, and Ser540 are relatively well conserved. A cyclin recognition motif and a Cdk substrate motif together constitute a bipartite substrate recognition sequence for cyclin-dependent kinases (Takeda *et al.* 2001). Ser540 fits the full consensus sequence for a Cdk substrate (S/T*-P-x-K/R), and Thr530 fits the minimal consensus sequence for a Cdk substrate (S/T*-P) (Songyang *et al.* 1994; Zhang *et al.* 1994; Srinivasan *et al.* 1995). Two cyclin recognition motifs (located between amino acid 203-207 and 519-523) are also well conserved. Some of the phosphorylation sites are not conserved because the N-terminal and the C-terminal domains are not present in orthologs of SIRT1 in lower organisms. Other sites that fit the minimal consensus sequence for Cdk substrates, such as Ser14, Ser26/27, Ser47 and Ser545, are only conserved in higher organisms.

Table II: Conservation of phosphorylated residues, cyclin recognition motifs, and Cdk substrate motifs among SIRT1 orthologs of 12 different species

Comparison of SIRT1 sequences by "BLAST 2 Sequence" among species, in which the protein sequence of the SIRT1 ortholog was available. Listed in order of the similarity to human SIRT1, from top to bottom. For species for which ELM motif data were available (BOLD), motif conservation was analyzed as well. For cyclin recognition motif, "ok" stands for a complete conservation of the motif, "?" for a partial conservation, and "ng" for loss of conservation. SP (14-15, 26-28, 47-48, 545-546) and TP (530-531) fit the minimal Cdk substrate motif, and the sequence between amino acids 537-543 fits the full Cdk substrate motif.

Abbreviations: cyc, cyclin recognition motif; Cdk, full Cdk-substrate motif.

Species	14	15	26	27	28	47	48	159	162	172	173	cyc 203-207	cyc 519-523	530	531	535	538	539	540	Cdk 537-543	544	545	546	719	747
H.sapiens	S	P	S	S	P	S	P	S	S	S	S	KDLLP	KELAY	T	P	S	S	S	S	DSS**SP**ER	T	S	P	T	S
P. troglodytes	S	P	S	S	P	S	P	S	S	S	S	KDLLP	KELAY	T	P	S	S	S	S	DSS**SP**ER	T	S	P	T	S
M. mulatta	S	P	S	P	P	S	P	S	S	S	S	KDDLP	KELAY	T	P	S	S	S	S	DSS**SP**ER	T	S	P	T	S
C. familiaris	S	P	S	P	P	S	P	S	S	S	S	KDDLP	KELAH(?)	T	P	S	S	S	S	DSS**SP**ER	T	S	P	I	S
B. Taurus	S	P	S	P	P	S	S	S	S	S	S	KDLLP	KELAH(?)	T	P	S	S	S	S	GSS**SP**ER	T	S	P	A	S
M. musculus	S	P	S	Q	P	S	P	S	S	S	S	KDLLP	KELV(ok)	T	P	S	S	S	S	DSS**SP**ER	T	V	P	A	S
R. norvegicus	L	G	A	G	R	D	P	S	S	S	S	KDLLP	KELV(ok)	T	P	S	S	S	S	DSS**SP**ER	T	V	P	A	S
G. gallus	G	P	#	A	E	E	D	S	S	S	S	KDLLP	KELEM(ok)	T	P	S	S	G	S	DSG**SP**EQ	M	T	P	E	#
D. melanogaster	#	#	#	#	#	#	#	G	N	S	S	ASIMP(ng)	KELMP(ok)	#	#	R	S	S	E	CSS**E**SER	Q	S	Q	#	#
C. elegans	#	#	#	#	#	#	#	#	#	#	#	QQIFP(ng)	RQLI(ok)	I	C	N	S	S	D	DSS**D**EPT	L	K	K	#	#
S. pombe	#	#	#	#	#	#	#	#	S	N	I	KKLGI(ok)	#	#	#	#	#	#	#	#	#	#	#	#	#
S. cerevisiae	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#

3.3.3 SIRT1 forms a complex with cyclin B and Cdk1 *in vivo*

Because cyclin recognition motifs and Cdk substrate motifs at Thr530 and Ser540 are well-conserved, and potential Cdk substrate residues Thr530 and Ser540 are phosphorylated *in vivo*, I hypothesized that SIRT1 could be a substrate for cyclin/Cdk. To test this hypothesis, I transfected 293T cells with a FLAG-SIRT1 expression vector and prepared cell lysates from cells in exponential growth phase. I immunoprecipitated SIRT1 using anti-FLAG M2 beads and analyzed the immune complexes by western blot. I found that Cdk1 co-precipitates with FLAG-SIRT1 (**Fig. 13a**). I did not detect co-precipitation of other Cdks involved in cell cycle regulation, namely Cdk2, Cdk4 and Cdk6. Cdk1 is a G2/M phase kinase that interacts with cyclin A and cyclin B to regulate mitosis (Nigg 2001). To determine which of these two cyclins were in the immune complex with SIRT1, I performed additional immunoprecipitations. I found that cyclin B1, but not cyclin A forms a complex with FLAG-SIRT1 (**Fig. 13a**). Furthermore, Cdk1 also co-precipitated with endogenous SIRT1 present in untransfected 293T cell lysates using polyclonal antibodies against N- and C-terminal fragments of SIRT1 (**Fig. 13b**), demonstrating that SIRT1 also forms a complex with cyclin B and Cdk1 *in vivo*.

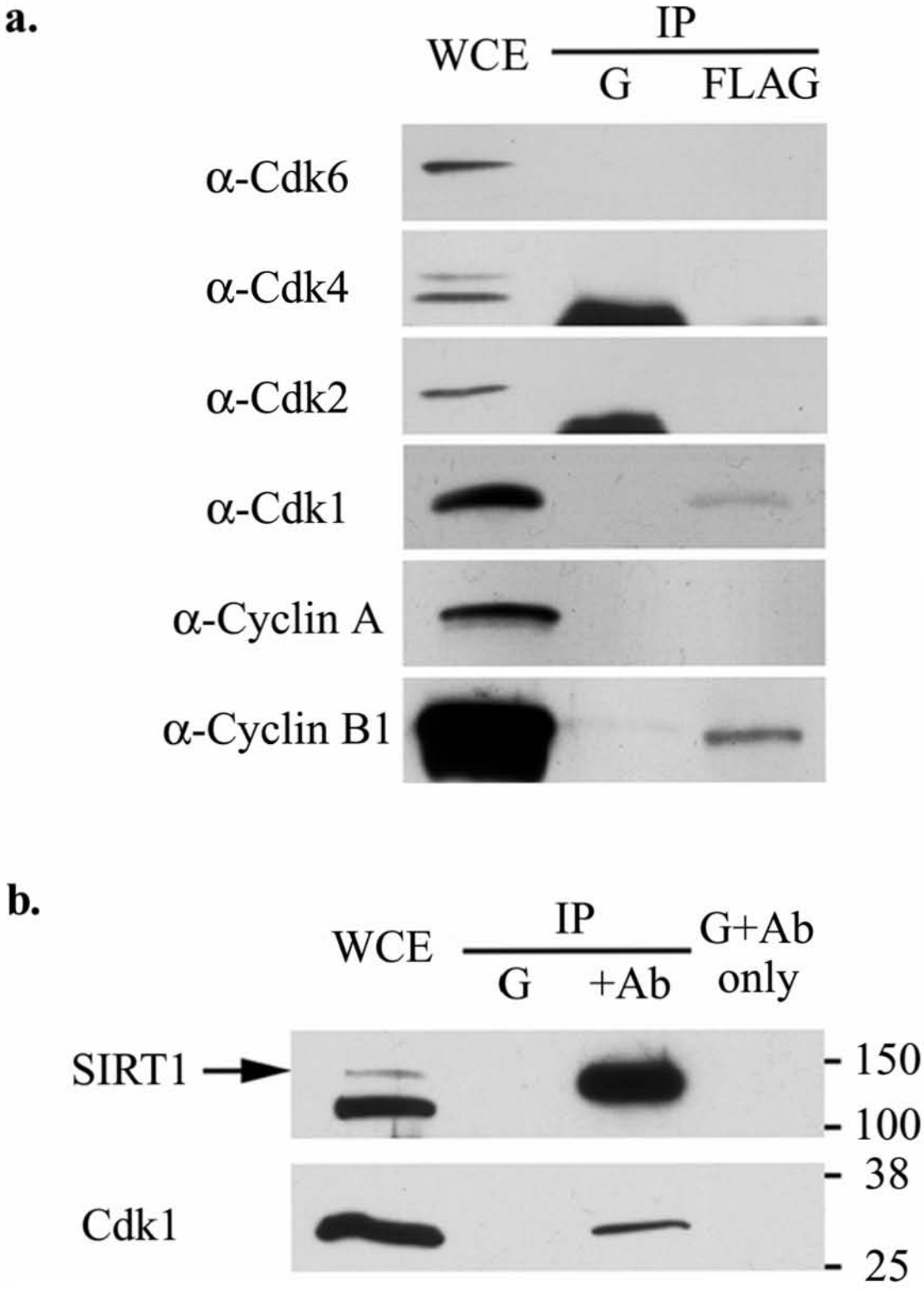

a.
WCE
IP
G
FLAG
α-Cdk6
α-Cdk4
α-Cdk2
α-Cdk1
α-Cyclin A
α-Cyclin B1
b.
WCE
IP
G
+Ab
G+Ab only
SIRT1
Cdk1
150
100
38
25

Figure 13. SIRT1 forms a complex with cyclin B/Cdk1

a. Western blot analysis of different Cdks and Cyclins following co-IP with FLAG-SIRT1. FLAG-SIRT1 forms a complex with Cdk1 and cyclin B, but not with Cdk2, Cdk4, Cdk6, or cyclin A. Amounts of samples used: WCE, 20μg; IP 500μg.

b. Western blot of co-IP experiments with endogenous SIRT1, which also forms a complex with Cdk1. Amounts of samples used: WCE, 20μg; IP 1.5mg.

WCE, whole cell extracts; IP, immunoprecipitation; G, protein G beads only; FLAG, FLAG-M2 beads; +Ab, protein G beads plus anti-SIRT1 antibodies; G+Ab only, protein G beads and anti-SIRT1 antibodies only, without cell lysate.

3.3.4 SIRT1 is phosphorylated by cyclin B/Cdk1 *in vitro*

To determine if cyclin B/Cdk1 could phosphorylate SIRT1 *in vitro*, I performed kinase assays using three different forms of SIRT1: affinity-purified human FLAG-SIRT1 and recombinant human SIRT1 with and without a GST-tag. Histone H1, a known substrate for cyclin B/Cdk1, was used as a positive control. I found that affinity-purified human FLAG-SIRT1 is a good substrate for phosphorylation by cyclin B/Cdk1 *in vitro* (**Fig. 14a, b**). However, recombinant human SIRT1 and GST-SIRT1 could not be phosphorylated by cyclin B/Cdk1 *in vitro*. To address this difference in substrate suitability for cyclin B/Cdk1, I tested several different hypotheses. One possibility was that unknown co-factor(s) necessary to make SIRT1 a suitable substrate for cyclin B/Cdk1 co-purified during affinity-purification of FLAG-SIRT1. These factors would be absent from preparations of recombinant SIRT1 proteins synthesized in bacteria. To test this hypothesis, preparations of FLAG-SIRT1 and GST-SIRT1 proteins were mixed prior to the *in vitro* kinase assays. As shown in **Fig. 14c**, however, only FLAG-SIRT1 (MW 120kD) was labeled by radioactive ATP in this assay, while GST-SIRT1 (MW 140kD) remained unlabeled. This result does not support the hypothesis that unknown co-factor(s) make SIRT1 a suitable substrate for cyclin B/Cdk1. The trace amount of kinase activity in affinity-purified FLAG-SIRT1 in the absence of added cyclin B/Cdk1 (**Fig. 14c**, lane 1 and 3) is due to cyclin B/Cdk1 co-purified with FLAG-SIRT1. Radio-labeling of FLAG-SIRT1 following incubation with ^{32}P γ-ATP overnight could be inhibited by a pan-Cdk inhibitor (olomoucine) and by a selective Cdk1 inhibitor (CGP74514A), but not by a GSK3 inhibitor (**Fig. 14d**).

Another possibility that could account for the difference in the ability of SIRT1 purified from mammalian cells and recombinant SIRT1 synthesized in bacteria to be phosphorylated by Cdk1 is that SIRT1 requires other post-translational modifications, such as phosphorylation at additional sites, to become a suitable substrate for cyclin B/Cdk1. If this were true, then prior dephosphorylation of SIRT1 should impair *de novo* phosphorylation by cyclin B/Cdk1. To address this possibility, I incubated affinity-purified FLAG-SIRT1 with increasing concentrations of λppase, then treated the dephosphorylated substrates with cyclin B/Cdk1. As shown in **Fig. 15a**, the ability of cyclin B/Cdk1 to phosphorylate SIRT1 decreased with increasing doses of λppase, and was abolished when SIRT1 was maximally dephosphorylated. To confirm these results, I performed a second *in vitro* kinase assay using radioactive ATP to detect protein phosphorylation by cyclin B/Cdk1. After treatment with higher doses of λppase, there was minimal incorporation of radioactive phosphate into FLAG-SIRT1 (**Fig. 15b, c**). At the same time, Histone H1 was efficiently labeled by radioactive ATP even at the highest doses of λppase. Finally, I used a mitotic kinase mix, which contains high cyclin B/Cdk1 activity as well as other mitotic phase kinases (Li *et al.* 2006), to phosphorylate FLAG-SIRT1 in the presence of radioactive ATP. As shown in **Fig. 15c**, in the presence of these additional kinases, SIRT1 was phosphorylated even at the highest doses of λppase, though to a lesser degree. These data imply that SIRT1 may require additional post-translational modification(s) to become a suitable substrate for cyclin B/Cdk1, and may be a substrate for other kinases as well.

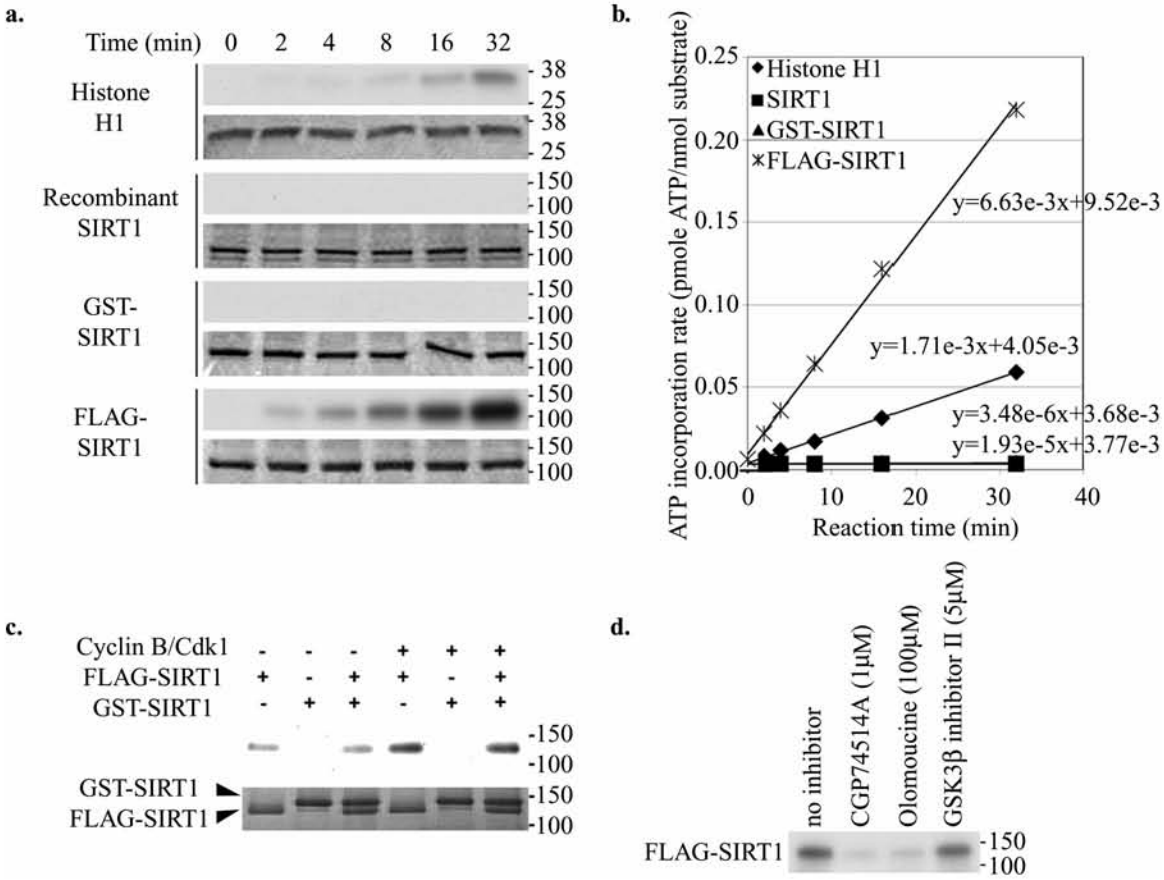

a.
Time (min) 0 2 4 8 16 32
Histone H1
Recombinant SIRT1
GST-SIRT1
FLAG-SIRT1
38
25
150
100
b.
Histone H1
SIRT1
GST-SIRT1
FLAG-SIRT1
y=6.63e-3x+9.52e-3
y=1.71e-3x+4.05e-3
y=3.48e-6x+3.68e-3
y=1.93e-5x+3.77e-3
ATP incorporation rate (pmole ATP/nmol substrate)
Reaction time (min)
c.
Cyclin B/Cdk1
FLAG-SIRT1
GST-SIRT1
GST-SIRT1
FLAG-SIRT1
d.
no inhibitor
CGP74514A (1μM)
Olomoucine (100μM)
GSK3β inhibitor II (5μM)
FLAG-SIRT1

Figure 14. SIRT1 is phosphorylated by cyclin B/Cdk1 *in vitro*

a/b. *In vitro* kinase assays with cyclin B/Cdk1. Affinity-purified FLAG-SIRT1 is a good substrate for cyclin B/Cdk1 *in vitro*, but recombinant SIRT1 with and without a GST tag are not.

a. Autoradiographs of radioactively labeled proteins separated by SDS-PAGE (upper panels) and pictures of Coomassie blue-stained gels (lower panels). Substrates are indicated to the left of each pair of panels.

b. ATP incorporation *vs.* time. ATP incorporation was determined by scintillation counting of the radioactivity in each band.

c. Autoradiograph and Coomassie blue-stained gel following *in vitro* kinase assays with mixture of affinity-purified FLAG-SIRT1 and recombinant GST-SIRT1. Only FLAG-SIRT1 (the lower band on Coomassie stain) is phosphorylated.

d. Autoradiograph of kinase assays in the presence of inhibitors.

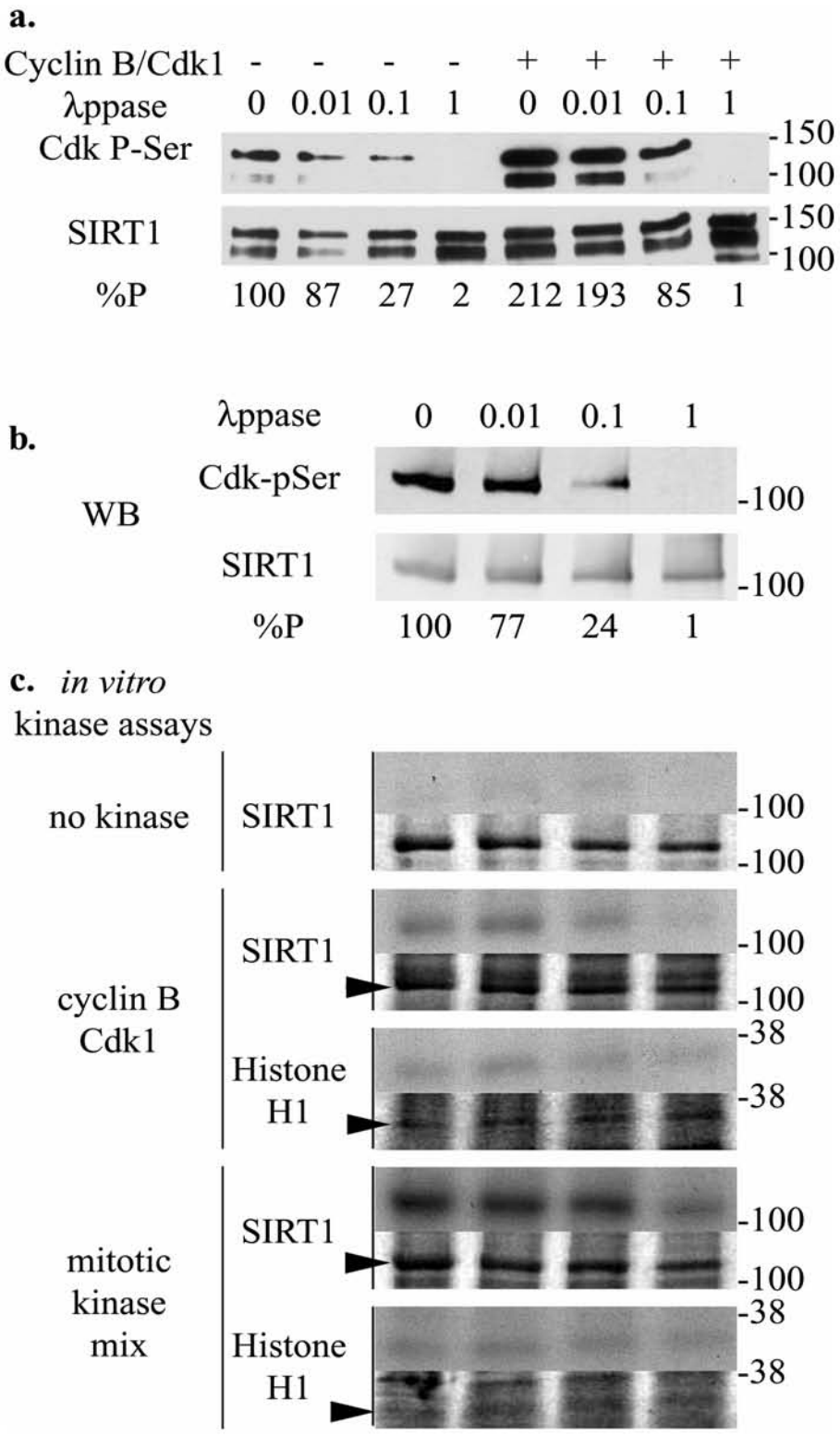

a.
Cyclin B/Cdk1 - - - - + + + +
λppase 0 0.01 0.1 1 0 0.01 0.1 1
Cdk P-Ser
-150
-100
SIRT1
-150
-100
%P 100 87 27 2 212 193 85 1
b.
λppase 0 0.01 0.1 1
WB
Cdk-pSer
-100
SIRT1
-100
%P 100 77 24 1
c. in vitro
kinase assays
no kinase
SIRT1
-100
-100
cyclin B
Cdk1
SIRT1
-100
-100
Histone
H1
-38
-38
mitotic
kinase
mix
SIRT1
-100
-100
Histone
H1
-38
-38

Figure 15. SIRT1 requires phosphorylation to become a suitable substrate for cyclin B/Cdk1 *in vitro*

a. Western blots of Cdk phosphorylated (top panel) and total SIRT1 (bottom panel) following treatment with increasing doses of lambda phosphatase (λppase).

b/c. Western blots of dephosphorylated SIRT1 (**b**) used for *in vitro* kinase assays (**c**). Autoradiograph (top panels) and Coomassie blue-stained gels (bottom panels) of SIRT1 phosphorylated by the kinases indicated on left. Histone H1 was spiked into kinase reaction to show that kinase activity is present.

0, 0.01, 0.1, 1, relative doses of λppase used; %P, relative percentage of Cdk phospho-serine signal compared to control.

3.3.5 Threonine 530 and serine 540 of SIRT1 are targets for cyclin B/Cdk1 *in vitro*

Based on the observations that SIRT1 is a substrate for cyclin B/Cdk1 *in vitro*, and that Thr530 and Ser540 are conserved potential substrates and phosphorylated *in vivo*, I hypothesized that these two residues could be substrates for cyclin B/Cdk1. To test this hypothesis, I changed one or both of them to a non-phosphorylatable amino acid (alanine), then tested whether or not the mutant proteins could be phosphorylated by cyclin B/Cdk1 *in vitro*. Compared to wild type, the Ser540→Ala (S540A) mutant showed approximately 50% reduction in the incorporation of radioactive ATP and the Thr530→Ala (T530A) and T530A/S540A double mutant (AA) showed approximately 35% reduction (**Fig. 16a, b**). This indicates that both Thr530 and Ser540 are substrates for cyclin B/Cdk1 *in vitro*. The fact that the radioactive signal decreased in the mutants, but did not go away completely, indicates that other less well-conserved residues that fit the minimal Cdk substrate motif, such as Ser14, Ser27, Ser47 and Ser545 (**Table II**) may also be substrates for cyclin B/Cdk1 *in vitro*.

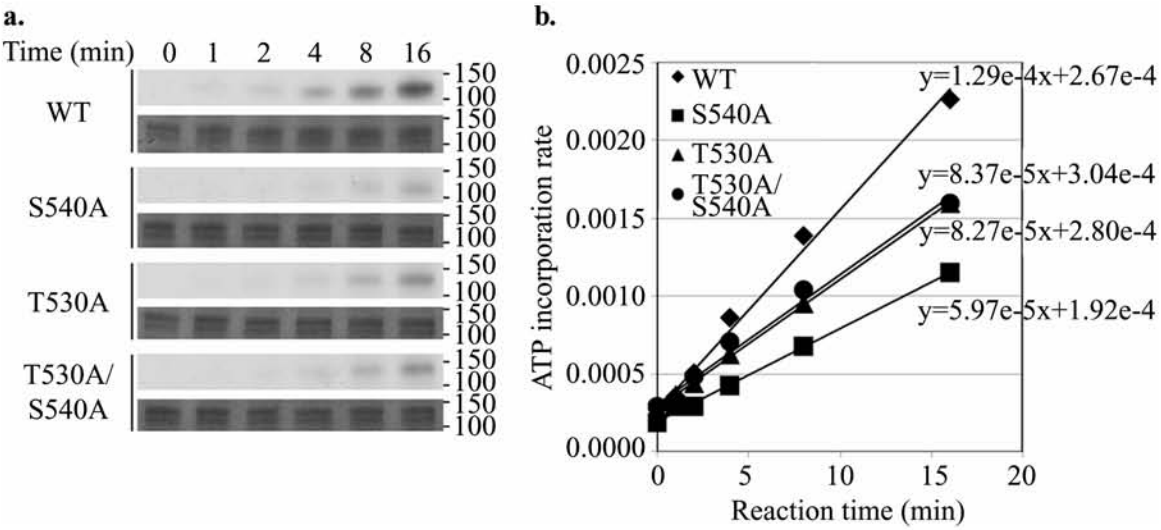

a.
Time (min) 0 1 2 4 8 16
WT
S540A
T530A
T530A/
S540A
150
100
150
100
b.
ATP incorporation rate
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
WT
S540A
T530A
T530A/
S540A
y=1.29e-4x+2.67e-4
y=8.37e-5x+3.04e-4
y=8.27e-5x+2.80e-4
y=5.97e-5x+1.92e-4
0
5
10
15
20
Reaction time (min)

Figure 16. Threonine 530 and serine 540 of SIRT1 are substrates for cyclin B/Cdk1 *in vitro*

a/b. *In vitro* kinase assays showing that threonine 530 and serine 540 are substrates for cyclin B/Cdk1 *in vitro*. The results are shown by autoradiograph (top panels) and Coomassie blue stained gel (bottom panels) in (**a**) and by the plot of ATP incorporation measured by scintillation countering *vs.* time in (**b**).

3.3.6 SIRT1 deacetylase activity is modulated by phosphorylation

After establishing that SIRT1 is a phosphoprotein and is a substrate for cyclin B/Cdk1, I looked into potential biological functions of SIRT1 phosphorylation. To test the possibility that the phosphorylation of SIRT1 might modulate its NAD^+-dependent deacetylase activity, I compared the enzymatic activity of phosphorylated and dephosphorylated affinity-purified FLAG-SIRT1 using a fluorogenic peptide-substrate-based assay system, *Fluor-de Lys* (Howitz *et al.* 2003). As shown in **Fig. 17a and b**, dephosphorylation led to a decline in deacetylase activity. Western blot analysis of the samples confirmed that the Cdk sites were dephosphorylated (bottom panel). Neither the deacetylase activity nor the reaction with the anti-Cdk phospho-serine antibody decreased when phosphatase activities were blocked by phosphatase inhibitors. Therefore, the general loss of SIRT1 phosphorylation leads to a decline in its deacetylase activity.

To rule out the possibility that changes in deacetylase activity were due to the activity of other classes of histone deacetylases, which are NAD^+-independent, contaminating the FLAG-SIRT1 preparation, I repeated the *Fluor-de Lys* deacetylase assay in the absence of NAD^+. As shown in **Fig. 17c**, there was no measurable deacetylase activity in the FLAG-SIRT1 preparation unless NAD^+ was added to the reaction.

To test the effect of Thr530/Ser540 phosphorylation on the deacetylase activity of SIRT1 *in vitro*, I compared the deacetylase activity in preparations of FLAG-SIRT1 with or without the T530A and/or S540A mutations. No significant differences in the NAD^+-dependent deacetylase activity were detected (**Fig. 17d**).

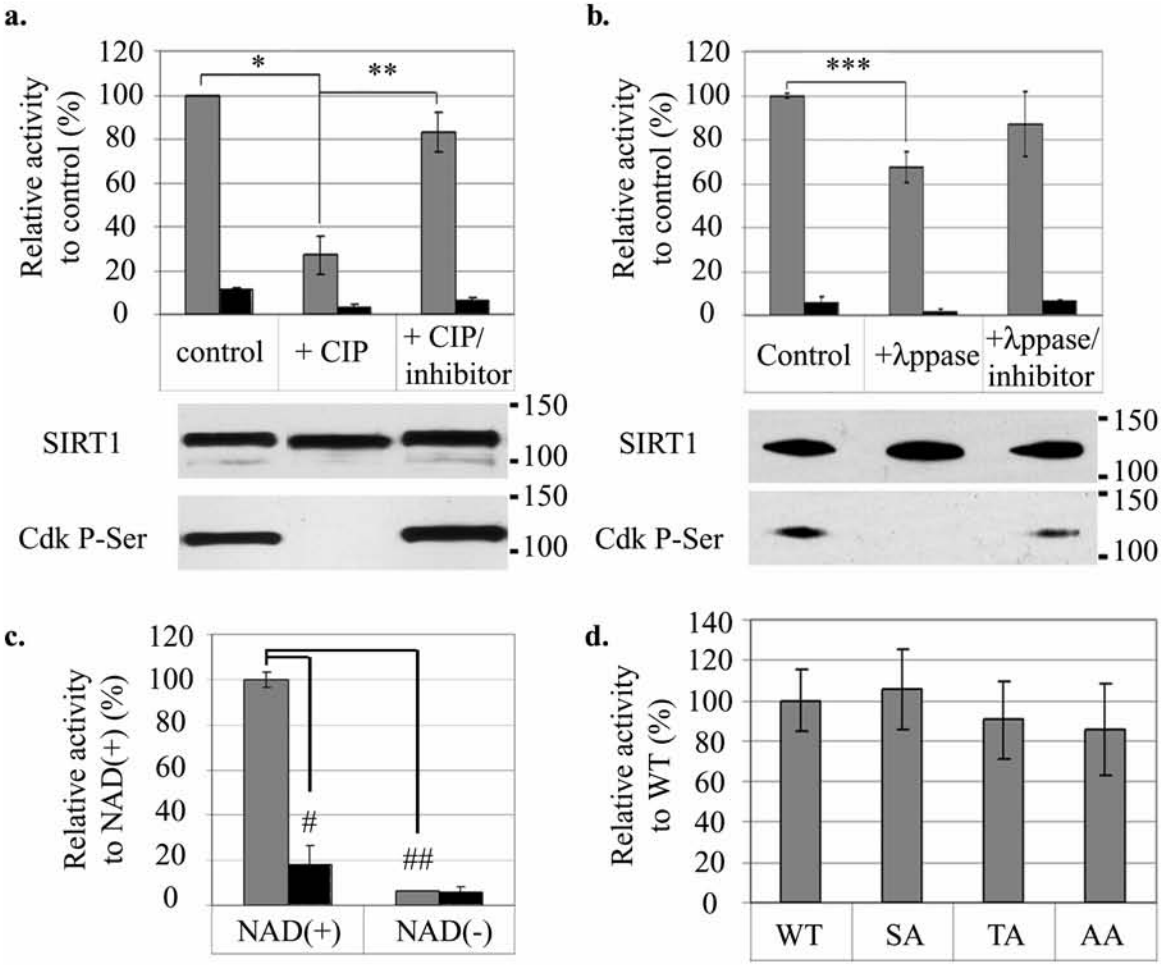

a.
Relative activity to control (%)
120
100
80
60
40
20
0
*
**
control
+ CIP
+ CIP/ inhibitor
SIRT1
Cdk P-Ser
150
100
150
100
b.
Relative activity to control (%)
120
100
80
60
40
20
0
***
Control
+λppase
+λppase/ inhibitor
SIRT1
Cdk P-Ser
150
100
150
100
c.
Relative activity to NAD(+) (%)
120
100
80
60
40
20
0
#
##
NAD(+)
NAD(-)
d.
Relative activity to WT (%)
140
120
100
80
60
40
20
0
WT
SA
TA
AA

Figure 17. NAD^+-dependent deacetylase activity of SIRT1 is modulated by phosphorylation

a/b. NAD^+-dependent deacetylase activity of SIRT1 in the presence of CIP (**a**) or λppase (**b**) with or without phosphatase inhibitors. Western blots below each bar graph indicate the amount of SIRT1 in the reaction (top panel) and the degree of SIRT1 phosphorylation represented by Cdk P-Ser signal (bottom panel).

c. Deacetylase activity of affinity-purified FLAG-SIRT1 in the presence (left) or absence (right) of NAD^+. Gray bars, no nicotinamide added; black bars, + nicotinamide.

d. NAD^+-dependent deacetylase activity of SIRT1 in wild-type (WT), S540A (SA), T530A (TA), and T530A/S540A double mutant (AA).

*, $p<0.005$; **, $p<0.02$; ***, $p<0.002$; #, $p<0.01$; ##, $p<0.001$ (Student's *t*-test). Error bars indicate +/- SEM.

3.3.7 Phosphorylation of SIRT1 at Thr530 and Ser540 influences cell proliferation and cell cycle profiles

To test the significance of SIRT1 phosphorylation at Thr530 and Ser540 on cell proliferation, I transfected wild-type or *Sirt1*-deficient ES cells with expression vectors for FLAG-SIRT1 with the T530A/S540A mutation (AA) or without mutation (WT) and measured cell proliferation. *Sirt1*-null embryos and mice are smaller than their wild-type counterparts (Cheng *et al.* 2003; McBurney *et al.* 2003), and this could be due to slower cell proliferation. I found that the proliferation defect of *Sirt1*$^{-/-}$ ES cells could be rescued by WT SIRT1, but not by the T530A/S540A double mutant (**Fig. 18a, b**). The same effect was seen in *Sirt1*$^{-/-}$ MEFs (**Fig. 18c, d**).

To determine which phase of the cell cycle was affected by loss of *Sirt1*, I compared the cell cycle profiles of wild-type and *Sirt1*-deficient ES cells. Cells were stained with propidium iodide and an anti-phospho-histone H3 (Ser10) antibody that is detected only during mitosis (Xu *et al.* 2001). I found that *Sirt1*$^{-/-}$ ES cells have a significantly higher portion of cells in S-phase than wild-type ES cells (**Figure 19a**). Finally, to determine how loss of Cdk1-dependent phosphorylation of SIRT1 affected the cell cycle, I transfected wild-type and *Sirt1*-deficient ES cells with expression vectors encoding wild-type or mutant (AA) SIRT1 and analyzed the cell cycle by FACS. I found that wild-type SIRT1 could rescue the increase in the number of *Sirt1*-deficient ES cells in S-phase, but the AA mutant could not. In fact, this non-phosphorylatable form of the protein acted as a dominant negative in *Sirt1*$^{+/+}$ ES cells, causing an increase in S-phase similar to that seen in *Sirt1*-deficient cells (**Fig. 19b**). Therefore, the AA mutation, which

results in the loss of Cdk1 substrate sites in SIRT1, affects cell proliferation and cell cycle profile.

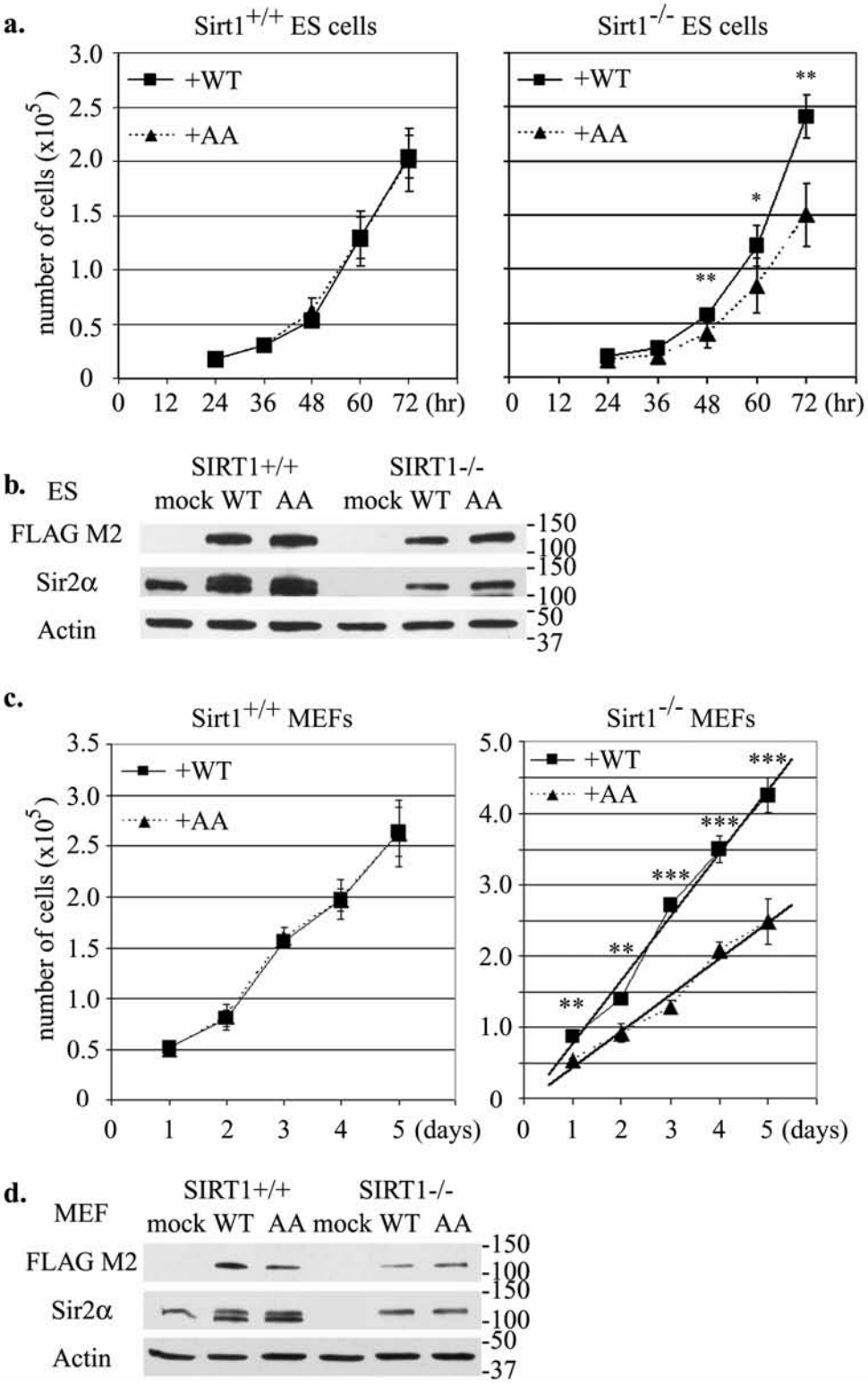

a.
Sirt1+/+ ES cells
Sirt1-/- ES cells
+WT
+AA
number of cells (x10^5)
3.0
2.5
2.0
1.5
1.0
0.5
0
0 12 24 36 48 60 72 (hr)
*
**
b.
ES
SIRT1+/+
SIRT1-/-
mock WT AA mock WT AA
FLAG M2
Sir2α
Actin
-150
-100
-150
-100
-50
-37
c.
Sirt1+/+ MEFs
Sirt1-/- MEFs
+WT
+AA
number of cells (x10^5)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
5.0
4.0
3.0
2.0
1.0
0
0 1 2 3 4 5 (days)
**
***
d.
MEF
SIRT1+/+
SIRT1-/-
mock WT AA mock WT AA
FLAG M2
Sir2α
Actin
-150
-100
-150
-100
-50
-37

Figure 18. Phosphorylation of SIRT1 at Thr530 and Ser540 affects cell proliferation

a/c. Proliferation assays in *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ ES cells (**a**) or MEFs (**c**) transfected with wild-type (WT) or mutant (AA) SIRT1.

b/d. Western blots showing expression levels of FLAG-SIRT1. Note that two bands are seen with the Sir2α antibody in *Sirt1*$^{+/+}$ ES cells transfected with FLAG-SIRT1. The upper band corresponds to FLAG-SIRT1 and the lower band to endogenous SIRT1.

*, $p<0.05$; **, $p<0.01$; ***, $p<0.002$ (One-way ANOVA followed by *t*-test with LSD procedure). Error bars indicate +/- SEM.

a.

	G1	S	G2	M
Sirt1+/+	25.0+/-1.2	58.7+/-0.8	15.1+/-1.2	1.2+/-0.2
Sirt1-/-	26.9+/-0.9	62.7+/-1.8	9.9+/-2.2	0.4+/-0.1
p	n.s.	0.04	n.s.	0.02

b.

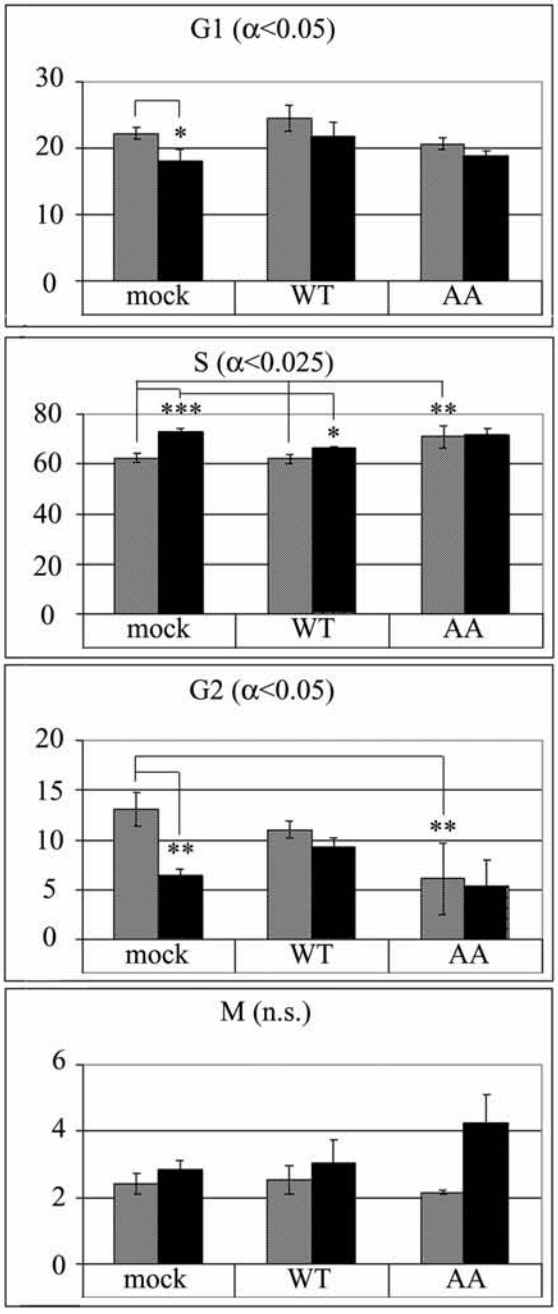


Figure 19. Thr530 and Ser540 are required for rescue of S-phase in Sirt1-deficient cells

a. Fraction of cells in each phase of the cell cycle in *Sirt1*$^{+/+}$ (n=6) and *Sirt1*$^{-/-}$ (n=3) ES cells. P values by Students' *t*-test.

b. Graphic representation of the fractions of cells in each phase of the cell cycle in *Sirt1*$^{+/+}$ (gray bars) and *Sirt1*$^{-/-}$ (black bars) ES cells transfected with empty vector (mock), WT-FLAG-SIRT1 (WT), or AA-FLAG-SIRT1 (AA) mutant. One-way ANOVA was applied to each cycle among 6 groups (n=3 each). Because the G1, S, and G2 phase fractions were significantly different among samples, they were analyzed using *t*-test with LSD procedure. WT was compared to mock of corresponding genotype, and AA was compared to mock and WT of corresponding genotype. *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ were compared in mock group only. Values are average +/- SEM. *, $p<0.05$; **, $p<0.02$; ***, $p<0.01$; n.s., not significant.

3.4 DISCUSSION

In this work, I have shown that SIRT1 is phosphorylated at 13 residues *in vivo* and is a substrate for cyclin B/Cdk1 *in vitro*. Furthermore, the phosphorylation of SIRT1 affects its deacetylase activity, cell proliferation, and cell cycle profile. Some of the phosphorylated residues, cyclin-recognition motifs, and Cdk substrate motifs are conserved among species. Analyses of the conservation of cyclin-recognition motifs and phosphorylated residues among species revealed an interesting aspect of these results. The residue that corresponds to Ser540 in human SIRT1 is E (glutamate) in *D. melanogaster* and D (aspartate) in *C. elegans*. S→D and S→E mutations are commonly used as phospho-mimetics, suggesting that, in these organisms that mainly consist of post-mitotic cells, this site acts like one that is permanently phosphorylated. In higher organisms that depend on cell proliferation for the life-long maintenance of tissue integrity, however, this region of SIRT1 has acquired the ability to interact with cyclin and Cdk and be regulated by cycles of phosphorylation and dephosphorylation.

Although this is the first extensive report on SIRT1 phosphorylation, other classes of HDACs are known to be regulated by phosphorylation. The activities and complex formation of HDAC1 and HDAC2 are regulated by phosphorylation (Pflum *et al.* 2001; de Ruijter *et al.* 2003), and nucleo-cytoplasmic localization of HDAC4, HDAC5, and HDAC7 are regulated by 14-3-3 in a phosphorylation-dependent manner (Grozinger and Schreiber 2000; Dequiedt *et al.* 2006). According to functional motif predictions obtained through the Eukaryotic Linear Motif database (Puntervoll *et al.* 2003), Ser26 and Ser27 of human SIRT1 are within interaction motif 2 of 14-3-3, and Ser172 and

Ser173 are within interaction motif 3. Thus, it is possible that the interaction of SIRT1 with 14-3-3, which has been demonstrated in *C. elegans* (Berdichevsky *et al.* 2006; Wang *et al.* 2006), is also regulated by phosphorylation in higher organisms.

The results of our study also imply that SIRT1 might be regulated by multiple hierarchical phosphorylations, similar to the situation with HDAC7, where phosphorylation of Ser 155 is a prerequisite for phosphorylation of Ser181 (Dequiedt *et al.* 2006). According to functional motif predictions obtained through ScanSite (http://scansite.mit.edu) (Obenauer *et al.* 2003), KinasePhos (http://kinasephos.mbc.nctu.edu.tw) (Huang *et al.* 2005), and Eukaryotic Linear Motifs (http://elm.eu.org) (Puntervoll *et al.* 2003) servers, the 13 phosphorylated residues of SIRT1 identified in this study match target motifs for ATM, Cdk5, CK1, CK2, DNA-PK, ERK1, GSK3, IKK and MAPK, in addition to Cdk1. Thus, any number of signaling cascades could influence SIRT1 function through the activity of various kinases and their accompanying phosphatases. It was recently reported for HDAC7, for example, that dephosphorylation by myosin phosphatase controls its nucleocytoplasmic shuttling and inhibits apoptosis in thymocytes (Parra *et al.* 2007). The large number of potential kinases and the redundancy of kinases that can act on a single site could explain why I did not detect any fluctuation in the phosphorylation of the Cdk sites of SIRT1 when I blocked cells in S phase or M phase with aphidicolin or nocodazole, respectively (data not shown).

When FLAG-SIRT1 was dephosphorylated by treatment with non-specific phosphatases, there was a concomitant decrease in deacetylase activity. Mutation of the

Cdk1 phosphorylation sites, however, did not lead to a reduction in deacetylase activity, at least as measured by the *Fluor-de Lys* deacetylase assay. It is possible that additional phosphorylation sites may have to be mutated to see the effect of loss of Cdk1 phosphorylation on deacetylase activity. Another possibility is that phosphorylation of certain residues may have positive influence on deacetylase activity, while others may have a negative or no influence. It should be noted that even relatively complete dephosphorylation by non-specific phosphatases did not totally abolish deacetylase activity. Considering the fact that all the phosphorylation sites are located outside of the conserved catalytic domain, it is also possible that phosphorylation of these residues may affect enzyme activity indirectly, for example, by altering complex formation with substrates, rather than directly. This could also explain why, using a fluorogenic peptide as a substrate rather than a full-length protein, I could not detect any difference in deacetylase activity between wild-type and mutant FLAG-SIRT1. A similar result was observed when SIRT2, another sirtuin that is phosphorylated by Cdk1, was mutated at the Cdk1 site. Using a histone peptide substrate *in vitro*, no decrease in enzyme activity was observed (North and Verdin 2007).

SIRT2, which is a cytoplasmic isoform of SIRT1, has been reported to be phosphorylated during G2/M transition at multiple sites and to be dephosphorylated by Cdc14a and Cdc14b phosphatases (Dryden *et al.* 2003; North and Verdin 2007). This, along with our findings, strengthens the association between sirtuins and cyclin-dependent kinases. Because SIRT2 phosphorylation may be linked to ubiquitination and subsequent degradation by the proteasome (Dryden *et al.* 2003), it is possible that SIRT1

phosphorylation, and possibly dephosphorylation by Cdc14b, may regulate its turnover as well. However, neither SIRT1 ubiquitination nor identification of a ubiquitin E3 ligase for SIRT1 have been reported.

The targets that SIRT1 deacetylates in the context of mitosis remain undefined. Although SIRT1 is predominantly a nuclear protein, it could potentially interact with cytoplasmic substrates during M phase, when the nuclear envelope breaks down. Cyclin B/Cdk1 kinase activity is tightly regulated and increases during M phase of the cell cycle, yet substrates vary and include proteins that regulate DNA replication, mitosis, spindle assembly, actin polarization, and other processes in yeast (Ubersax *et al.* 2003). SIRT1 substrates in the context of mitosis may also function during other phases of cell cycle, for example during S-phase, as suggested by our results. The initiation of DNA replication is tightly regulated in eukaryotic cells to ensure the precise duplication of DNA once (and only once) per cell division cycle. DNA replication occurs in two discrete steps, replication "licensing" and "initiation" (reviewed in (Arias and Walter 2007)). During the first step (replication licensing), prereplication complexes (pre-RC) are formed on replication origins, beginning as cells exit M phase and continuing during G1 phase. The assembly of pre-RC at each origin involves the ordered binding of at least four factors, ORC, Cdc6, Cdt1, and Mcm2-7. ORC binds to origin DNA and recruits Cdc6 and Cdt1. Eventually, the minichromosome maintenance complexes (Mcm2-7) are recruited to origins and complete the formation of pre-RC. Recent studies suggest a potential role of chromatin structure in the control of DNA replication (Simpson 1990; Brown *et al.* 1991; Lipford and Bell 2001; Vogelauer *et al.* 2002; Aparicio *et al.* 2004).

Histone acetylation is involved in origin activation at the chorion gene loci in *Drosophila* follicle cells (Aggarwal and Calvi 2004), and in *Xenopus* early development (Danis *et al.* 2004). Hbo1, a MYST family histone acetyltransferase (HAT) (Utley and Cote 2003), also positively regulates pre-RC assembly, possibly by influencing Mcm2-7 loading (Iizuka *et al.* 2006). Mutation of the acetylated lysine residues in histone H4 results in a prolonged S phase (Megee *et al.* 1990). On the other hand, among the histone deacetylases, only Sir2 (a class III HDAC) has been shown to inhibit pre-RC assembly in budding yeast (Pappas *et al.* 2004). Human SIRT1 deacetylates H4 lysine 16, H3 lysine 9, and H1 lysine 26, which promote formation of facultative heterochromatin (Vaquero *et al.* 2004). Because pre-RC assembly starts soon after cyclin B/Cdk1 activity drops (Arias and Walter 2007), it is possible that SIRT1 is phosphorylated by cyclin B/Cdk1 and that this phosphorylation is retained and influences SIRT1 function in the context of pre-RC assembly. Such a model could explain why Sirt1-deficient ES cells have slower growth characteristics despite accumulating in S-phase, and how transfection of FLAG-SIRT1 can rescue this effect. It could also explain the dominant effect of the SIRT1-AA mutant on S-phase in cells with normal Sirt1. Loss of the two key Cdk1 target residues would result in loss of cyclin B/Cdk1 control of SIRT1 and unregulated deacetylase activity at inappropriate points in the cell cycle.

3.5 Experimental procedures

3.5.1 Cell culture

Sirt1$^{-/-}$ and Sirt1$^{+/+}$ ES cells (gifts from Dr. F. Alt, Harvard) were cultured on gelatin-coated plates in DMEM supplemented with 15% FBS, L-glutamine, non-essential amino acids, nucleosides, β-mercaptoethanol, murine leukemia inhibitory factor (LIF) (all from Millipore, Billerica, CA) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). ES cells were cultured in the absence of feeder cells to get an accurate number for cell counting. Sirt1$^{-/-}$ and Sirt1$^{+/+}$ MEFs were prepared from E12.5 embryos generated by intercrossing Sirt$^{+/-}$ mice. 293T cells were purchased from ATCC (Manassas, VA). 293T cells and MEFs were cultured as previously described (Sasaki *et al.* 2006).

3.5.2 Expression vector construction and transfection

SIRT1 mutant expression vectors were generated by PCR mutagenesis using the SIRT1-expression vector pcDNA4/TO/FLAG-SIRT1 (a gift from Dr. D. Reinberg, NYU) (Vaquero *et al.* 2004) as a template. The CMV promoter of the pcDNA4/TO/FLAG-SIRT1 vector was replaced with the Pgk promoter at MluI/EcoRV sites to allow the expression of FLAG-SIRT1 in ES cells. For the empty pPGK vector, the pPGK-FLAGSIRT1 vector was digested with EcoRV and PmeI to remove FLAG-SIRT1 cDNA, and then blunt-end ligated. Plasmids were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

3.5.3 Protein preparation and immunoprecipitation

Total cell lysates were prepared as previously described (Sasaki *et al.* 2006). Immunoprecipitations of total cell lysates were performed with FLAG-M2 beads (Sigma-Aldrich), or anti-SIRT1 N-terminal polyclonal antibody (Millipore #07-131), anti-SIRT1 C-terminal polyclonal antibody AS-16 (Sigma-Aldrich #S5313) and protein G agarose (Millipore) for 2 hours or overnight. After washing 3 times with lysis buffer, SDS-PAGE and western blot analysis were performed. FLAG-SIRT1 protein was purified by incubating Benzonase-treated (Sigma-Aldrich) total cell lysates with anti-FLAG M2 beads for 2 hours or overnight, then washed 3 times with wash buffer (10% glycerol, 50mM Tris pH 7, 150mM NaCl), then eluted with FLAG peptide (Sigma-Aldrich). Further gel filtration was performed using HiLoad 6/16 Superdex 200 column and AKTA FPLC system (GE Healthcare, Piscataway, NJ) when necessary. Samples were concentrated by centrifugation through Microcon YM-10 filters (Millipore). GST-SIRT1 protein was purified as previously described using BL21-CodonPlus competent cells (Stratagene, La Jolla, CA) and the GST-SIRT1 expression vector pGEX2TK-SIRT1 (a gift from Dr. J. Smith, UVA) (Gallo *et al.* 2004). The purity of the proteins was tested by western blot and Coomassie blue staining, and the activities were measured using the *Fluor-de-Lys* deacetylase assay kit (Biomol International, Plymouth Meeting, PA).

3.5.4 Western blot

Samples were separated by SDS-PAGE using 10% or 4-20% Tris-HCl gels and transferred using standard protocols. To detect the antigen-antibody complexes, I used

Western Pico or Femto chemiluminescence substrate (Pierce, Rockford, IL). The following antibodies were used: Peroxidase-conjugated AffiniPure Goat-anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA, #115-035-100, 1:10,000), Peroxidase-conjugated AffiniPure Goat anti-Rabbit IgG (Jackson ImmunoResearch #110-035-144, 1:10,000), anti-Cdc2 (Cdk1) mAb (Santa Cruz Biotechnology, Santa Cruz, CA, sc-8395, 1:1,000), anti-Cdk2 pAb (Abcam, Cambridge, MA, ab7954, 1:1,000), anti-Cdk4 pAb (Abcam ab2945, 1:1,000), anti-Cdk6 mAb (Abcam ab3126, 1:1,000), anti-cyclin A pAb (Abcam ab7956, 1:1,000), anti-cyclin B1 pAb (Abcam ab7957, 1:1,000), anti-FLAG M2 mAb (Sigma-Aldrich F3165, 1:2,000), anti-SIRT1 N-terminal polyclonal antibody (Millipore #07-131, 1:1,000), anti-SIRT1 C-terminal polyclonal antibody AS-16 (Sigma-Aldrich #S5313, 1:1,000), anti-SIRT1 monoclonal antibody 2G1/F7 (Millipore #05-707, 1:1,000) and anti-Cdk phospho-serine antibody (Cell Signaling Technology, Danvers, MA, #2324, 1:2,000). For **Fig. 15b**, the ODYSSEY infrared imaging system (LI-COR, Lincoln, NE) was used to quantitate the percentage of phosphorylated SIRT1 in λppase-treated samples.

3.5.5 Evolutionary conservation analysis of SIRT1 amino acid sequences

Amino acid sequences of SIRT1 from various species were obtained from the NCBI database, and subjected to the BLAST 2 Sequence algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) against the human SIRT1 sequence. The following sequences were used for the analysis: *Homo sapiens* (human, NP_036370), *Pan troglodytes* (chimpanzee, XP_521490), *Macaca mulatta* (macaque monkey,

XP_001087854), *Canis familiaris* (domestic dog, XP_546130), *Bos taurus* (cow, XP_869911), *Mus musculus* (mouse, AAR23928), *Rattus norvegicus* (rat, XP_001080493), *Gallus gallus* (chicken, NP_001004767), *Drosophila melanogaster* (fruit fly, AAC79684), *Caenorhabditis elegans* (round worm, CAA94364), *Schizosaccharomyces pombe* (fission yeast, CAG47122) and *Saccharomyces cerevisiae* (budding yeast, NP_010242).

3.5.6 ProQ Diamond phospho-protein staining

After protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane, the membrane was stained with Pro-Q Diamond phosphoprotein staining kit (Invitrogen P-33356) following the manufacturer's protocol.

3.5.7 Phospho-peptide mapping by mass spectrometry

Phospho-peptide mapping was done as previously described (Abbas *et al.* 2007). The gel band containing FLAG-SIRT1 was identified by silver stain, cut out, and the peptides generated by trypsin treatment. Peptides were analyzed using the LC-MS system.

3.5.8 Phosphatase prep and treatment

Solutions of calf intestinal phosphatase (New England Biolab, Ipswich, MA) or lambda phosphatase (a gift from Dr. D. Brautigan, UVA) were prepared and incubated with FLAG-SIRT1 samples at 1:1 volume for 1 hour at 37°C unless otherwise indicated.

Phosphatase activity was blocked by adding phosphatase inhibitor cocktails I & II (Sigma-Aldrich) or 10mM Na_3VO_4 (Sigma-Aldrich).

3.5.9 *In vitro* kinase assay

Kinase reactions were performed in 20mM Tris HCl (pH 7.5), 1mM $MgCl_2$, 25mM KCl, 1mM DTT, and 40μg/mL BSA, with 100μM of ATP spiked with γ-^{32}P-ATP. Cyclin B/Cdk1 purified from *Xenopus* was used with 5-10 pmole of substrate. Samples were harvested at each time point, and reactions quenched with 6X-sample buffer. Samples were separated by SDS-PAGE and stained with Bio-Safe Coomassie (Biorad). Gels were photographed with ChemiImager Ready (IMGEN technologies, Alexandria, VA). After autoradiography, each protein band was cut out of the gel and the radioactivity in the sample measured using a Beckman LS 6000SE scintillation counter (Beckman Coulter, Fullerton, CA). Kinase inhibitors used were olomoucine (EMD Biosciences, San Diego, CA), CGP74514A (EMD Biosciences), and GSK3β inhibitor II (EMD Biosciences). "Mitotic kinase mix" (a gift from Dr. D. Brautigan, UVA) was prepared according to the protocol described in reference (Li *et al.* 2006).

3.5.10 *Fluor-de Lys* deacetylase assay

Fluor-de Lys deacetylase assay kit (Biomol International) was used to analyze deacetylase activities in various SIRT1 preparations, using a slight modification of the protocol recommended by the supplier to adjust for the detection range of the fluorometer. Nicotinamide was added to the reaction at the concentration of 2mM when necessary.

The final fluorescence was measured in a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific, Waltham, MA).

3.5.11 Cell proliferation assay

Lipofectamine 2000 (Invitrogen) was used to transfect 1 x 10^6 ES cells with 2μg of empty vector or expression vectors for FLAG-SIRT1 with or without the T530A and S540A double mutation. All vectors carried the PGK-promoter to support expression in ES-cells. 24 hours after transfection, 2 x 10^4 cells/well were re-plated onto gelatin-coated 12-well plates, and cells were counted 24, 36, 48, 60, and 72 hours after replating. Each group consisted of triplicates and the experiment was done three times, and the average and the standard error of the number of cells in each well were calculated. For MEFs, 5 x 10^4 cells/well at passage 3 (split at 1:4 at each passage, used prior to immortalization) were transfected with vectors driven by the CMV promoter, and done in triplicates.

3.5.12 Cell cycle analysis

Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at a concentration of 10^6 cells per ml at -20°C for 2 hours or overnight. After fixation, cells were stained with propidium iodide (Sigma-Aldrich) and anti-phospho histone H3 (Ser10) mitosis marker antibody (Millipore, 1:1,000) followed by Alexa 488-conjugated goat anti-rabbit immunoglobulin G antibody (Invitrogen, 1:2,000), as previously described (Xu *et al.* 2001). Analyses were done at the University of Virginia Flow Cytometry Facility and cell cycle distributions were

analyzed using ModiFit LT version 3.1 software (Verity Software House, Topsham, ME). The number of cells in the G2 phase of the cell cycle was calculated by subtracting the number of cells in M phase (determined by phospho-H3 staining) from the number of cells in G2/M phase (determined by PI staining).

3.6 Acknowledgements

I thank Dr. Danny Reinberg (Howard Hughes Medical Institute, Smilow Research Center, Department of Biochemistry, New York University, New York, NY) for pcDNA4/TO/FLAG-SIRT1, Dr. Fredrick Alt (Howard Hughes Medical Institute, Department of Genetics, Harvard University, Boston, MA) for $Sirt1^{+/+}$ and $Sirt1^{-/-}$ ES cells, Dr. Jeffrey S. Smith (Department of Biochemistry and Molecular Genetics at the University of Virginia) for pGEX2TK-SIRT1 and Dr. Scott Zeitlin (Department of Neuroscience, University of Virginia) for the Pgk-Neo vector. I thank Dr. David Brautigan (Center for Cell Signaling at the University of Virginia) for providing lambda phosphatase and mitotic kinase mix and for many helpful discussions. I thank Dr. Thomas Sturgill (Department of Pharmacology, University of Virginia) for thoughtful discussions. I thank Dr. Jeremy Tuttle (Department of Neuroscience at the University of Virginia) for the use of Fluoroskan Ascent FL and Dr. Terry Turner (Department of Urology at the University of Virginia) for the use of Eppendorf Centrifuge 5810R. I thank the mass spectrometry core facility at the University of Virginia for phospho-peptide mapping of FLAG-SIRT1 protein and the flow cytometry core facility in the Department of Microbiology at the University of Virginia for their service.

This work was supported by PHS grant NIH AG026094 (H.S.), GM53163 (W.M.) and GM74942 (W.M.). H.S. is a fellow of the Ellison Foundation for Medical Research. The flow cytometry core facility is supported by Cancer Center Support Grant P30 CA45579.

Chapter 4: Possible roles and significance of SIRT1 phosphorylation

4.1 Summary

This dissertation began with defining SIRT1 and its significance in aging research; regulation of SIRT1 and cell cycle events were also discussed. This background provided the framework for the experiments reported in Chapter 2. These studies involved investigating the relationship between SIRT1 and mitotic activity of cells in the context of aging (*in vivo*) and senescence (*in vitro*), which had not previously been explored. I found that as mitotic activity ceases in murine and human cells *in vivo* or *in vitro*, SIRT1 protein levels concomitantly decline, a decrease that occurs post-transcriptionally. Based on these findings and the prediction that SIRT1 contains cyclin and Cdk recognition motifs, I tested the hypothesis in Chapter 3 that SIRT1 is regulated by phosphorylation conferred by Cdks. These studies, detailed in Chapter 3, revealed that SIRT1 is a phosphoprotein *in vivo* and is phosphorylated by Cyclin B/Cdk1 at Thr530 and Ser540 *in vitro*. Moreover, SIRT1 phosphorylation affects its deacetylase activity as well as cell proliferation and cell cycle profile.

In this chapter, I will discuss the potential significance of SIRT1 phosphorylation on SIRT1 function and its possible impact on biological processes, particularly cell cycle progression.

4.2 SIRT1 phosphorylation and deacetylase activity

When phosphorylation of FLAG-SIRT1 was decreased by phosphatase treatment, the deacetylase activity decreased concomitantly (**Fig. 17a, b**). The degree of the decline in deacetylase activity differed according to the phosphatase used (either CIP or λppase); it also varied among the λppase-treated groups. SIRT1 was shown to be phosphorylated at 13 serine/theronine residues, as detected by mass spectrometry, raising the possibility that phosphorylation of each residue could have positive, negative, or neutral effects on SIRT1 deacetylase activity. Each λppase treatment could have resulted in different degrees of dephosphorylation of the 13 sites, which could possibly cause variations in the reduction of deacetylase activity. Determining the effects of phosphorylation at each residue on deacetylase activity would require detailed mutagenesis studies, combining mutations of serine or threonine to alanine, which prohibits phosphorylation of the residue, and of serine or threonine to aspartate or glutamate (phosphomimetic mutations).

The 13 phosphorylated residues found in SIRT1 are all located outside of the conserved core catalytic domain for deacetylase activity (**Table I, Fig. 12b**). Based on a prediction model of SIRT1 structure (by Bernhard Maier, personal communication), the phosphorylated residues are located on the surface of SIRT1, but not close to the catalytic groove (**Fig. 20**). Nonetheless, the cluster of phosphorylated sites is present at the junction of the core and C-terminal domains. It is possible that phosphorylation of SIRT1 could affect its conformation and the ability of protein substrates to access its catalytic groove (**Fig. 20a**). In my experiments, I might not have detected a difference in accessibility to the catalytic groove because deacetylase activity was measured by a

fluorogenic peptide substrate (**Fig. 17**), which could have easier access to the groove than full-length protein substrates. In fact, an analysis of the crystal structure of the SIRT2 homologue Hst2p indicated that the enzymatic activity of this sirtuin can be regulated by intra-molecular interactions (Zhao *et al.* 2003).

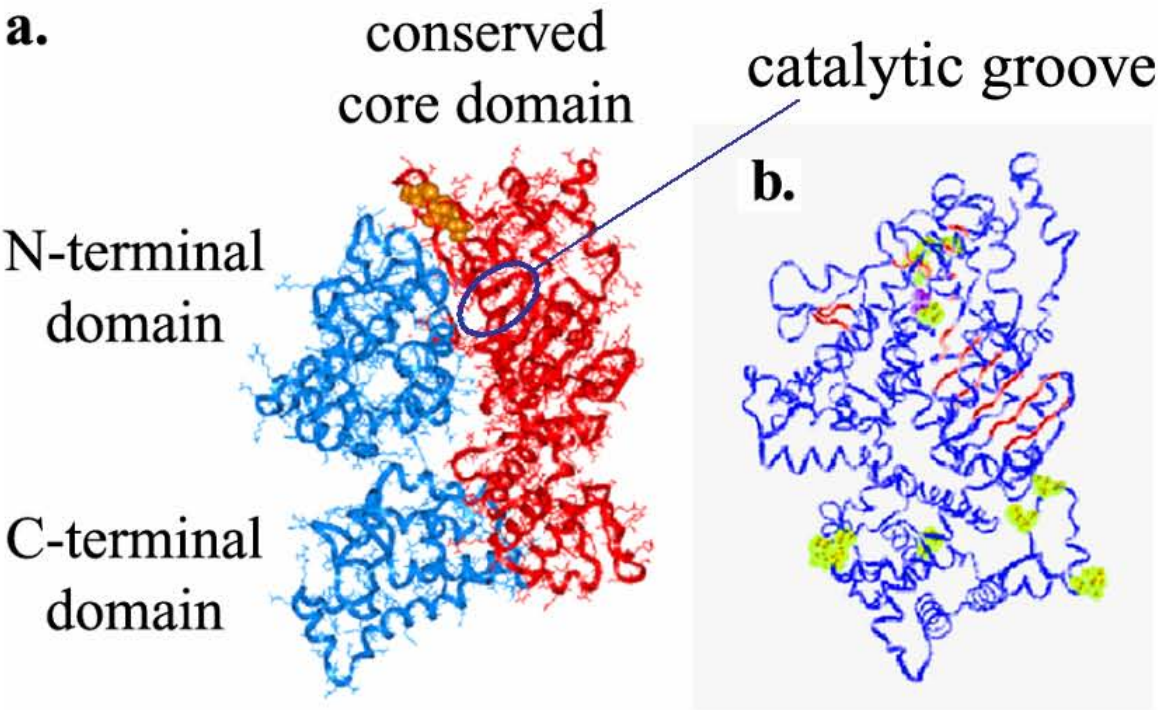

a.
conserved
core domain
catalytic groove
N-terminal
domain
C-terminal
domain
b.

Figure 20. Calculated 3D model of human SIRT1

a. Conserved core domain is shown in red.

b. Phosphorylated residues are marked by green. The orientation is slightly rotated from the position in **a.**

Bernhard Maier, personal communication.

4.3 SIRT1 phosphorylation and protein-protein interactions (SIRT1-FoxO/14-3-3 interaction)

Based on the argument above, it is also possible that phosphorylation of SIRT1 could affect its interactions with binding partners by changes in the protein's conformation. In fact, it is known that the activity and complex formation of other classes of HDACs are regulated by phosphorylation (Pflum *et al.* 2001; de Ruijter *et al.* 2003). Furthermore, nucleocytoplasmic localization of HDAC4, HDAC5, and HDAC7 are regulated by the 14-3-3 protein in a phosphorylation-dependent manner (Grozinger and Schreiber 2000; Dequiedt *et al.* 2006). In *C. elegans*, the 14-3-3 protein interacts with sir-2.1, an ortholog of SIRT1, and daf-16/FoxO (Berdichevsky *et al.* 2006; Wang *et al.* 2006). As predicted using Eukaryotic Linear Motifs (http://elm.eu.org) (Puntervoll *et al.* 2003), SIRT1 has two 14-3-3 interaction motifs and three forkhead-associated domains. Four out of thirteen phosphorylated residues found in SIRT1 are located within these motifs. Ser26 and Ser27 are within the 14-3-3 protein interaction motif 2, and Ser172 and Ser173 are within the 14-3-3 protein interaction motif 3. It is possible that interaction of human SIRT1 with FoxO and 14-3-3 proteins could also be regulated by phosphorylation. Predicted forkhead association domains are at amino acids 95-98, 344-347, and 614-617; phosphorylation of threonine residues is at the beginning of these domains and is a prerequisite for forkhead binding. Although I did not find phosphorylation of threonine residues using mass spectrometry, these sites could be functional forkhead association domains.

4.4 SIRT1 localization

SIRT1 is thought to be a nuclear protein, but cytoplasmic localization of SIRT1 has been reported (Moynihan *et al.* 2005; Chen *et al.* 2006). SIRT1 is predominantly shuttled to the nucleus by CRM1 (Tanno *et al.* 2007). Two nuclear localization signals are located in amino acids 31-38 and 223-230 of mouse Sirt1 (corresponding to amino acids 32-39 and 231-238 of human SIRT1) and two nuclear export signals are located in amino acids 138-145 and 425-431 of mouse Sirt1 (corresponding to amino acids 146-153 and 433-439 of human SIRT1). These sites are not associated with the phosphorylation sites detected by mass spectrometry in this study. In fact, I did not detect any difference in the localization of wild-type FLAG-SIRT1 and T530A/S540A mutant FLAG-SIRT1 between the nucleus and cytoplasm by fractionation or western blot analysis (**Fig. 21**).

As discussed previously, the 14-3-3 protein regulates nucleocytoplasmic localization of HDAC 4, HDAC5, and HDAC7 in a phosphorylation-dependent manner (Grozinger and Schreiber 2000; Dequiedt *et al.* 2006). Thus, it is possible that SIRT1 could be kept predominantly in the nucleus by a CRM-1 mediated mechanism and shuttled in and out of the nucleus via a 14-3-3 protein-mediated mechanism based on signals from various kinases and phosphatases.

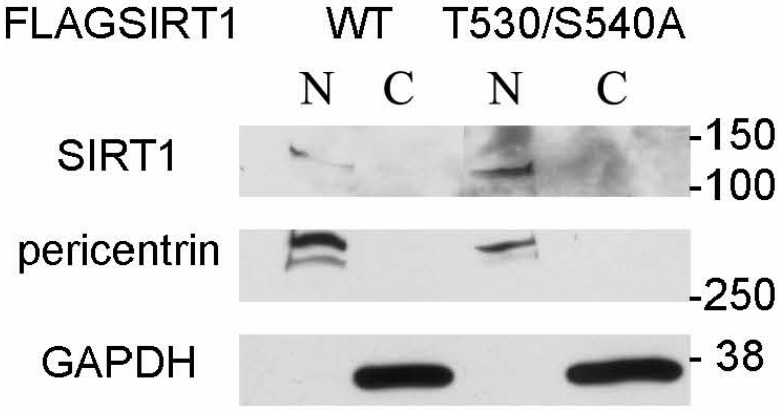

FLAGSIRT1
WT
T530/S540A
N
C
N
C
SIRT1
pericentrin
GAPDH
-150
-100
-250
- 38

Figure 21. Both WT and T530/S540A mutant SIRT1 are located in the nucleus

293T cells transfected with FLAG-SIRT1 expression vectors were fractionated between the cytoplasmic (C) and nuclear (N) compartment using cell lysis by NP-40 followed by SDS. Western immunoblotting was used to identify the FLAG-SIRT1 protein using FLAG M2 antibody (top), pericentrin (middle, nuclear marker) and GAPDH (bottom, cytoplasmic marker).

4.5 SIRT1 protein turnover

SIRT2, which is a cytoplasmic isoform of SIRT1, is phosphorylated at multiple sites during the G2/M transition of the cell cycle and dephosphorylated by the Cdc14b phosphatase (Dryden *et al.* 2003). This result, along with my finding that SIRT1 is phosphorylated by Cyclin B/Cdk1 (see Chapter 3), implies that sirtuins could be generally regulated by Cdks. Because SIRT2 phosphorylation is linked to ubiquitination and subsequent degradation by the proteasome (Dryden *et al.* 2003), it is possible that SIRT1 phosphorylation could also regulate its turnover but this possibility requires further investigation. A recent report showed that SIRT2 is phosphorylated on serine 368 by Cdk1 and dephosphorylated by Cdc14a and Cdc14b. However, they did not find SIRT2 degradation by the 26S proteasome in response to Cdc14b overexpression (North and Verdin 2007). Therefore, whether or not the turnover of sirtuins is regulated by the 26S proteasome is unclear.

Mass spectrometry data from my studies indicated that the upper band of the SIRT1 doublet detected by western blotting is ubiquitinated (**Figs. 5C&D, 7C, 9A, and 11** for murine samples and **Fig. 7A** for human samples). I also performed immunoprecipitation of FLAG-SIRT1 utilizing FLAG-M2 beads followed by western blotting with an anti-ubiquitin antibody, and I detected a faint ubiquitin signal (**Fig. 22a**).

Based on predictions made using Eukaryotic Linear Motifs (http://elm.eu.org) (Puntervoll *et al.* 2003), SIRT1 contains a COP1 recognition sequence. COP1 is an E3 ubiquitin ligase, and one of its substrates is p53 (Dornan *et al.* 2004). Another ubiquitin E3 ligase is NEDD4, which recognizes substrates with a WW4 domain; the recognition

of WW4 is dependent on phosphorylation of serine or theronine residue within the domain (Ingham *et al.* 2004). Residue S540 of SIRT1 fits the WW4 domain structure, as predicted. I tested if NEDD4 forms a complex with FLAG-SIRT1 using co-immunoprecipitation. I did not detect a NEDD4 signal in the sample immunoprecipitated by FLAG-M2 beads (**Fig. 22b**). At present, an E3 ubiquitin ligase for SIRT1 has not been reported. SIRT1 has been shown to be cleaved by caspase during apoptosis (Ohsawa and Miura 2006); however, it is not clear if SIRT1 phosphorylation would modulate its susceptibility to caspase cleavage.

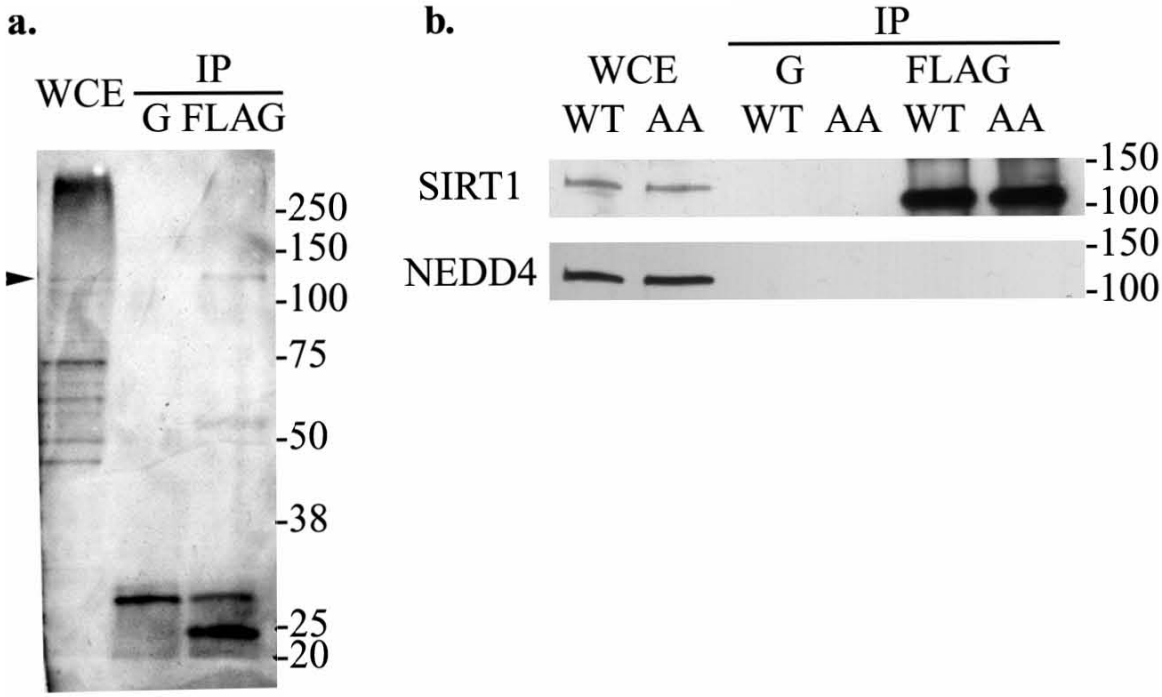

a.
WCE
IP
G FLAG
-250
-150
-100
-75
-50
-38
-25
-20
b.
IP
WCE
G
FLAG
WT AA WT AA WT AA
SIRT1
NEDD4
-150
-100
-150
-100

Figure 22. SIRT1 is ubiquitinated, but does not interact with NEDD4

a. IP/western blot of FLAG-SIRT1 with anti-ubiquitin antibody. The band corresponding to FLAG-SIRT1 is marked by an arrowhead.

b. FLAG-SIRT1 does not co-precipitate NEDD4. SIRT1-null cells were transfected with FLAG-SIRT1 expression vectors (with and without AA mutation) and IP/western blot performed.

Abbreviations: WCE, whole cell extract; IP, immunoprecipitation; G, protein G beads; FLAG, FLAG M2 bead.

4.6 Kinases that could potentially phosphorylate SIRT1

Based on the results discussed in Chapter 3 (**Figs. 15, 16**), SIRT1 may be regulated by multiple hierarchical phosphorylations, as reported for other classes of HDACs. For example, phosphorylation of Ser155 is prerequisite for phosphorylation of Ser181 in HDAC7 (Dequiedt *et al.* 2006). Based on predictions made for kinase motifs using ScanSite (http://scansite.mit.edu) (Obenauer *et al.* 2003), Eukaryotic Linear Motifs (http://elm.eu.org) (Puntervoll *et al.* 2003), and KinasePhos (http://kinasephos.mbc.nctu.edu.tw) (Huang *et al.* 2005), phosphorylated residues of SIRT1 identified in this study match target motifs for ATM, CDK5, CK1, CK2, DNA-PK, ERK1, glycogen synthase kinase 3 (GSK3), IKK and MAPK, as well as Cdk1 (**Table III**). Thus, combination of sites phosphorylated by various signaling cascades could influence SIRT1 function.

Examples of functionally interacting combinations of phosphorylated sites include CK2 and Cdk1 on the substrates p53 or high mobility group A protein (HMGA). p53 is phosphorylated by CK2 at Ser392, which interferes with its phosphorylation by Cdk1 at Ser315 (Wagner *et al.* 1998). CK2 also phosphorylates HMGA, which alters its conformation. It modulates the DNA binding properties of HMGA such that a subsequent phosphorylation by Cdk1 changes the organization of the protein-DNA complex (Schwanbeck *et al.* 2001). Nuclear-cytoplasmic localization of some proteins, such as pro-interleukin-16 and mSTI1, are regulated by a functional motif called CcN, which is comprised of the Cdk1 and CK2 substrate sites and the nuclear localization signal (Wilson *et al.* 2002; Longshaw *et al.* 2004).

Because S539 (CK2) and S540 (Cdk1) are adjacent, they may influence phorphorylation of the other residues. To test this idea, I first determined if SIRT1 is phosphorylated by CK2 *in vitro*. I found that CK2 can efficiently phosphorylate both recombinant GST-SIRT1 and affinity-purified FLAG-SIRT1 *in vitro* (**Fig. 23a**). However, pre-treatment of GST-SIRT1 with CK2 failed to make the protein a suitable substrate for Cyclin B/Cdk1 (**Fig. 23b, c**). Furthermore, the serine 539 to alanine (S539A) mutant did not show any decrease in ATP incorporation with CK2 treatment, indicating that this residue is not a suitable substrate for CK2 *in vitro* (**Fig. 23d**). Therefore, phosphorylation by CK2 is not a prerequisite for SIRT1 to be a suitable substrate for Cyclin B/Cdk1. I also did not detect the signals from CK2 subunits CK2α or CK2β by performing co-immunoprecipitation with FLAG-SIRT1 using FLAG-M2 beads (data not shown).

It is still possible, however, that other kinases may be able to phosphorylate SIRT1 thus allowing recognition by Cyclin B/Cdk1.

Table III. List of kinases that have recognition motifs that match the phosphorylated residues in SIRT1

Combined results for the human, mouse, and rat SIRT1 sequence from ScanSite, Eukaryotic linear motifs, and KinasePhos.

Abbreviations: ATM, ataxia-telangiectasia mutated; Cdk, cyclin-dependent kinase; CK1, casein kinase 1; CK2, casein kinase 2; DNA-PK, DNA-dependent protein kinase; ERK1, extracellular signal-regulated kinase 1; GSK3, glycogen synthase kinase-3; IKK, I kappa B kinase; MAPK, mitogen-activated protein kinase; PDK, proline-dependent kinase; and PKA, protein kinase A.

S14	ATM, Cdk1, Cdk5, PDK
S26	Cdk
S27	Cdk1, ERK1, GSK3, ERK1
S47	ATM, Cdk1, Cdk5, CK2, PDK
S159	CK1
S162	CK1, CK2, DNA-PK
S172	
S173	CK1, GSK3
T530	Cdk1, ERK1, MAPK, PDK
S535	ATM, CK1, CK2, GSK3
S538	CK2
S539	CK1, CK2
S540	ATM, Cdk, Cdk1, Cdk5, CK1, CK2, GSK3, IKK, PDK
T544	GSK3
S545	Cdk1, Cdk5, ERK1, GSK3, IKK, PDK, PKA
T719	CK2
S747	

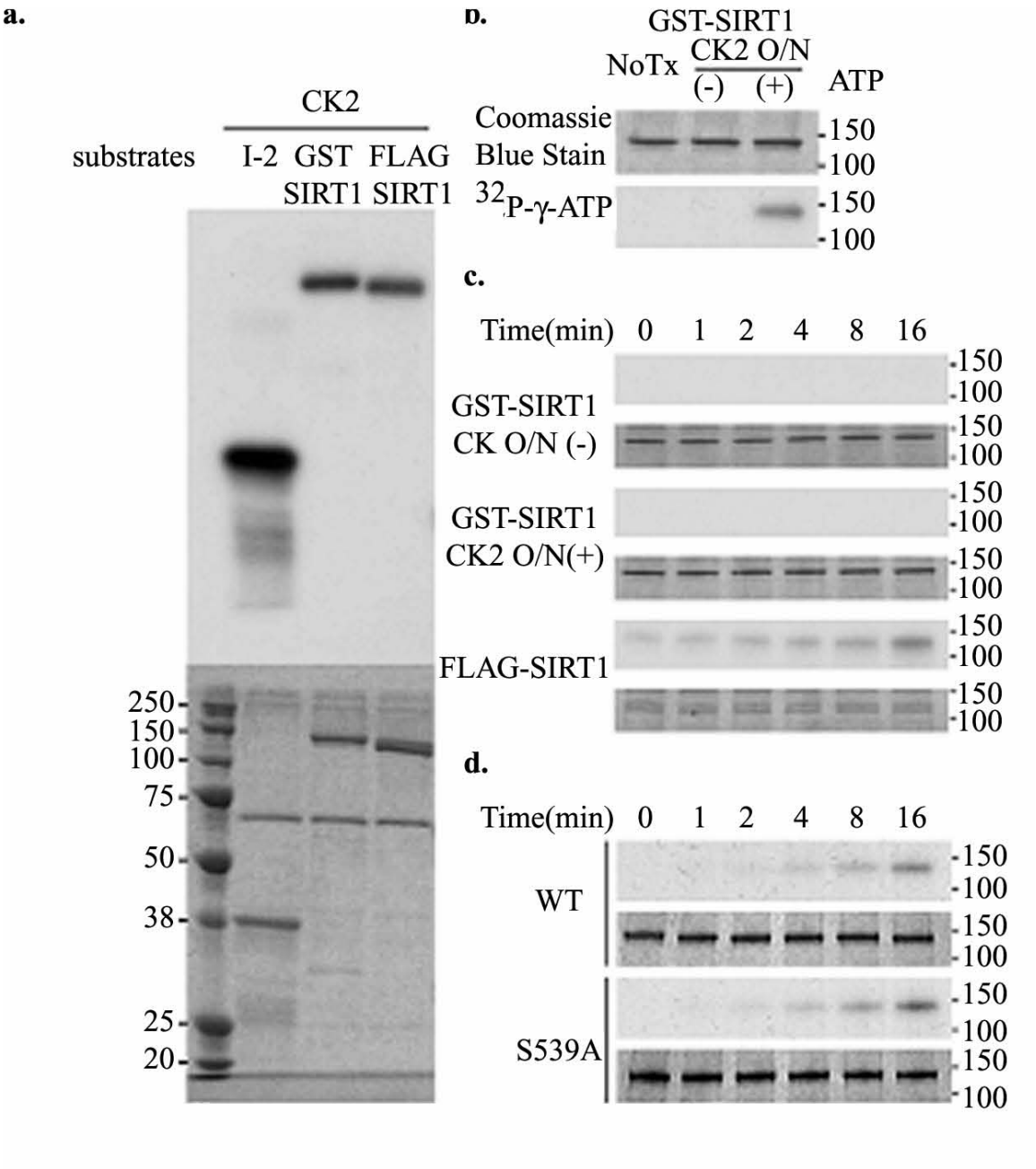

a.
CK2
substrates
I-2
GST SIRT1
FLAG SIRT1
250
150
100
75
50
38
25
20
b.
GST-SIRT1
NoTx
CK2 O/N
(-)
(+)
ATP
Coomassie Blue Stain
32P-γ-ATP
150
100
c.
Time(min) 0 1 2 4 8 16
GST-SIRT1 CK O/N (-)
GST-SIRT1 CK2 O/N(+)
FLAG-SIRT1
d.
Time(min) 0 1 2 4 8 16
WT
S539A

Figure 23. CK2 phosphorylates SIRT1, but does not provide the phosphorylation necessary to make recombinant SIRT1 a substrate for Cyclin B/Cdk1

a. *In vitro* kinase assay with CK2. Recombinant GST-SIRT1 and affinity-purified FLAG-SIRT1 are both phosphorylated by CK2. I-2 (a gift from Dr. David Brautigan) is a known substrate for CK2.

b. Overnight treatment of GST-SIRT1 with CK2 phosphorylates GST-SIRT1 without degrading the protein.

c. Overnight treatment with CK2 does not turn GST-SIRT1 into a Cyclin B/Cdk1 substrate. GST-SIRT1 was treated with CK2 in the same condition as **b.** in the absence of radioactive ATP. Then it was purified and used for the *in vitro* kinase assay with Cyclin B/Cdk1.

d. S539 of SIRT1 is not a substrate for CK2. *In vitro* kinase assay performed with wild-type and S539A mutant GST-SIRT1. There is no difference in the rate of ATP incorporation between the two samples.

4.7 Possible biological effects of SIRT1 phosphorylation based on its known substrates

My work focused mainly on the relationship between SIRT1 and mitotic activity of cells. However, as discussed above, many other kinases could phosphorylate SIRT1 and modulate its function. Furthermore, SIRT1 possesses a large and growing list of substrates (Blander and Guarente 2004), and could modulate numerous physiological functions, as discussed in Chapter 1. It is possible that SIRT1 could be a focal point in summarizing information from various signaling cascades via the phosphorylation of different sites. This in turn would alter deacetylase activity, and change the acetylation of substrate proteins, that modulate stress resistance, cell survival and differentiation, and glucose and lipid metabolism.

4.8 Possible regulation of cell cycle progression by SIRT1

Targets for deacetylation by SIRT1 in the context of mitosis remain unclear. Although SIRT1 is predominantly a nuclear protein, once the nuclear envelope breaks down for mitosis, it could also interact with cytoplasmic substrates (**Fig. 24**). Cyclin B/Cdk1 kinase activity is tightly regulated and increases during the M-phase of cell cycle, yet substrates of Cyclin B/Cdk1 vary, from proteins that regulate Cdk1, DNA replication, mitosis, spindle assembly, and actin polarization, to those that are involved in other processes not related to the cell cycle (Ubersax *et al.* 2003).

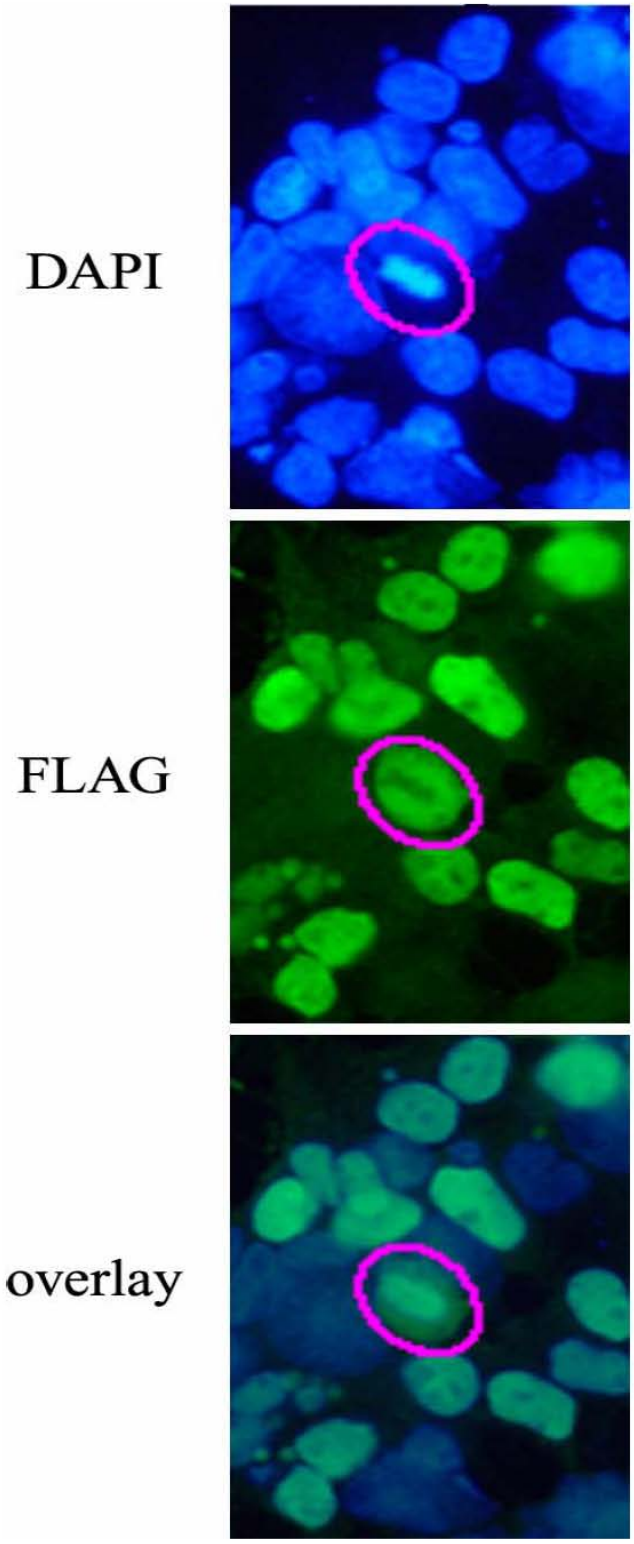

DAPI
FLAG
overlay

Figure 24. FLAG-SIRT1 is detected in cytoplasm when cells are in mitosis

293T cells were transfected with FLAG-SIRT1 expression vector, and then plated on glass slides, fixed and immunocytochemistry performed with anti-FLAG M2 monoclonal antibody and DAPI staining. A cell in mitosis (in pink circle) shows condensed DNA (DAPI staining, upper panel), while FLAG-SIRT1 is detected in the cytoplasm (FLAG staining, middle panel). The bottom panel is an overlay of the above two.

One possible substrate for SIRT1 is the α- and β-tubulin heterodimers that comprise the microtubule network. This network plays an important role in the regulation of cell shape, intracellular transport, cell motility, and cell division (Nogales 2000). Tubulin acetylation occurs on Lys40 of the α-tubulin subunit (Piperno *et al.* 1987). Microtubules are acetylated in domains that turn over slowly (Webster and Borisy 1989) and are associated with stable structures, such as cilia, that contain relatively hyperacetylated α-tubulin (Poole *et al.* 2001). During *Drosophila* embryogenesis, acetylated α-tubulin was detected in interphase microtubule arrays, but not in cells during mitosis (Wolf *et al.* 1988). During mouse fertilization and early development, microtubules show a cell-cycle-specific pattern of acetylation, with acetylated microtubules found at the centrosomes at metaphase, an increase in spindle labeling at anaphase, and selective deacetylation of all but midbody microtubules at telophase (Schatten *et al.* 1988). The function of α-tubulin is regulated by acetylation (Webster and Borisy 1989), but it is a more suitable substrate for SIRT2, not SIRT1 (North *et al.* 2003). I did not detect any difference in the acetylation of α-tubulin among *Sirt1*$^{+/+}$ or *Sirt1*$^{-/-}$ ES cells with and without transfection of wild-type or T530/S540A mutant FLAG-SIRT1 (**Fig. 25**). Therefore it is unlikely that SIRT1 regulates cell cycle progression through tubulin deacetylation.

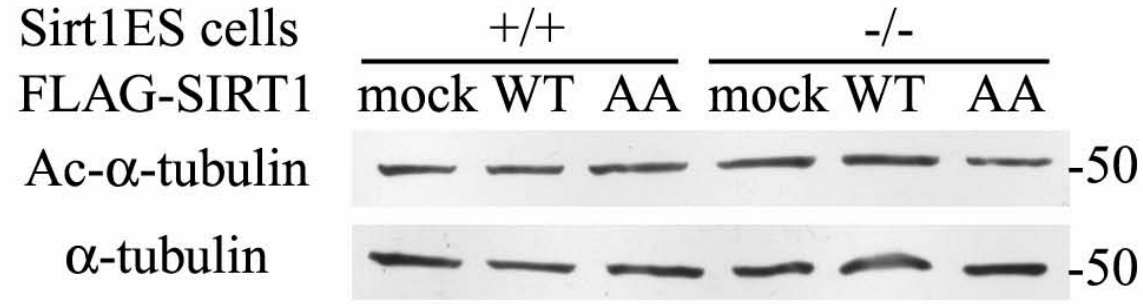

Sirt1ES cells
+/+
-/-
FLAG-SIRT1
mock WT AA mock WT AA
Ac-α-tubulin
-50
α-tubulin
-50

Figure 25. Acetylation of α -tubulin is not affected by the presence of SIRT1 or by T530A/S540A (AA) mutant

Sirt1$^{+/+}$ or *Sirt1*$^{-/-}$ ES cells were transfected with a mock vector, wild-type FLAG-SIRT1 expression vector (WT), or AA mutant expression vector, and analyzed using western blotting with an anti-acetylated lysine 40 α-tubulin monoclonal antibody (top row) or by anti α -tubulin monoclonal antibody (DM1α, bottom row).

The organization of DNA into chromatin and chromosomal structures plays a central role in many aspects of cell biology in eukaryotes. DNA, along with chromatin and other modifications, must be replicated during mitosis (Ehrenhofer-Murray 2004). Because SIRT1 deacetylates histones (Imai *et al.* 2000), the demand for SIRT1 may increase in proliferating cells. Then, as cells stop dividing, the demand for SIRT1 would decrease and the level of the protein would decline accordingly. It is possible that once DNA replication, including replication of chromatin modifications, are completed at the end of S-phase, Cyclin B/Cdk1 may modulate SIRT1 activity or its localization at M-phase to adjust to the needs of the cell.

The initiation of DNA replication is tightly regulated in eukaryotic cells to ensure the precise duplication of DNA once per cell division cycle. DNA replication occurs in two discrete steps, which are closely correlated with oscillations in Cdk activity. Two steps are replication "licensing" and "initiation" (**Fig. 26**, reviewed in (Arias and Walter 2007)). During the first step (replication licensing), pre-replication complexes (pre-RC) are formed on replication origins, beginning as cells exit M phase and continuing during the G1 phase. The assembly of pre-RC at each origin involves the ordered binding of at least four factors, ORC, Cdc6, Cdt1, and minichromosome maintenance complexes 2-7 (Mcm2-7). ORC binds to the origin DNA and recruits Cdc6 and Cdt1. Eventually, the Mcm2-7 are recruited to the origins and complete the formation of pre-RC. The second step (initiation of DNA replication) is triggered by an increase in S-phase Cdk activity, which occurs at the G1/S transition. S-phase Cdk activity and Cdc7 kinase activate the

pre-RC, converting it into an initiation complex. Once the pre-RC is activated, its reassembly is prohibited throughout S phase until the end of M phase.

A major mechanism for preventing the formation of the pre-RC involves the action of cyclin-dependent kinase, which negatively regulates the components of the licensing system. In multicellular animals with differentiated tissue (metazoans), an additional licensing inhibitor, geminin, also acts to prevent pre-RC assembly by specifically binding to Cdt1 and inhibiting its function.

Despite conservation of the proteins involved in eukaryotic DNA replication, the DNA sequences on which initiation takes place (origin DNA) are highly divergent, and in many organisms, poorly characterized (Gilbert 2001; Cvetic and Walter 2005). Metazoan ORC complexes exhibit virtually no sequence specificity (Vashee *et al.* 2003; Remus *et al.* 2004). However, replication initiation events in these organisms are not random, and can in some cases be quite localized.

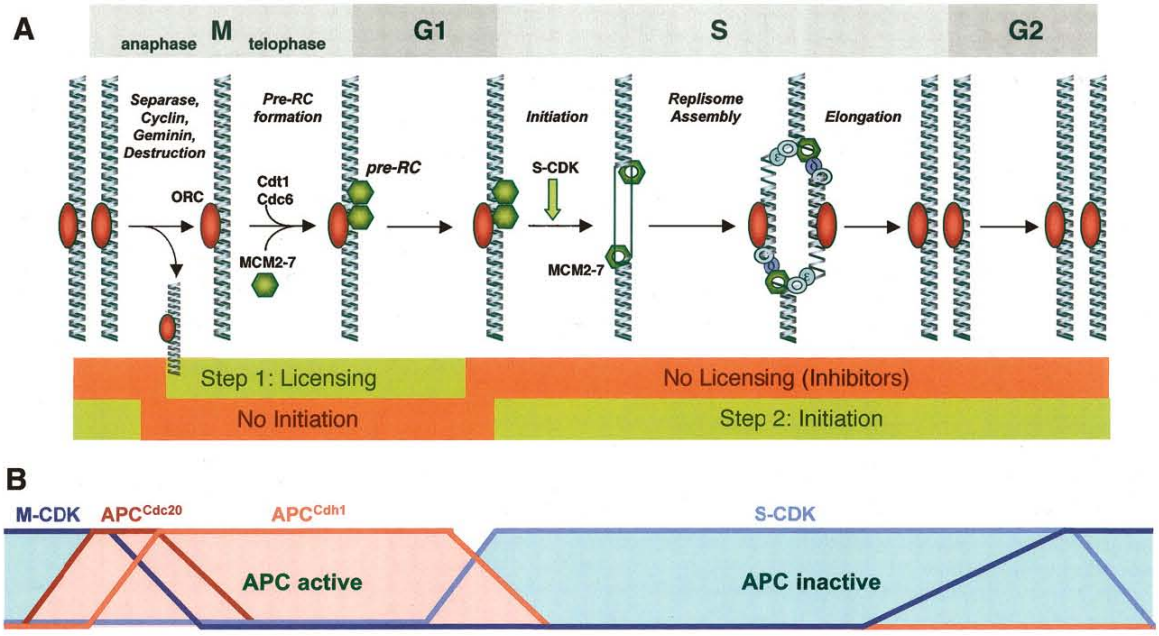

A
anaphase
M
telophase
G1
S
G2
Separase, Cyclin, Geminin, Destruction
Pre-RC formation
Initiation
Replisome Assembly
Elongation
ORC
Cdt1
Cdc6
MCM2-7
pre-RC
S-CDK
MCM2-7
Step 1: Licensing
No Licensing (Inhibitors)
No Initiation
Step 2: Initiation
B
M-CDK
APC^Cdc20
APC^Cdh1
S-CDK
APC active
APC inactive

Figure 26. Two-step model for the cell cycle regulation of eukaryotic DNA replication

A. The events that occur at origins of DNA replication at different stages of the cell cycle. The green bars indicate when in the cell cycle licensing and initiation, respectively, are allowed.

B. Oscillations in APC and Cdk activity during the cell cycle are indicated.

From (Arias and Walter 2007). Refer to section 4.8 for a detailed description.

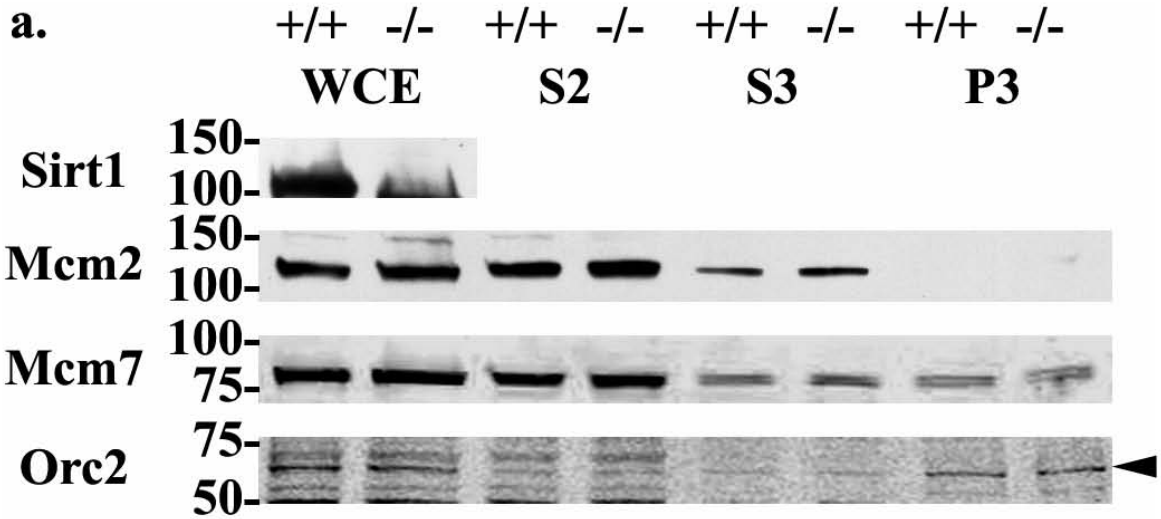
Recent studies suggest a potential role of chromatin structure in the control of DNA replication. Chromatin structure has been linked to the initiation of DNA replication (Simpson 1990; Brown *et al.* 1991; Lipford and Bell 2001), and histone modification has been implicated in regulating replication timing in budding yeast (Vogelauer *et al.* 2002; Aparicio *et al.* 2004). Histone acetylation is involved in origin activation at the chorion gene loci in *Drosophila* follicle cells (Aggarwal and Calvi 2004), and in *Xenopus* early development (Danis *et al.* 2004). Acetylated histones are localized to active origins at amplification foci, coincident with ORC. Conversely, tethering of deacetylases to the DNA leads to a decrease in origin activity. Hbo1, a MYST family histone acetyltransferase (HAT) (Utley and Cote 2003), also positively regulates pre-RC assembly, possibly by influencing Mcm2-7 loading (Iizuka *et al.* 2006). Mutations of the acetylated lysine residues in histone H4 results in a prolonged S phase (Megee *et al.* 1990).

On the other hand, SIR2 (a class III HDAC), but not RPD3 (class I HDAC) or HDA1 (a class II HDAC), in budding yeast has been shown to supress pre-RC assembly, but not subsequent steps of DNA replication (Pappas *et al.* 2004). Human SIRT1 deacetylates histone H4 lysine 16, H3 lysine 9, and H1 at lysine 26, which promote formation of facultative heterochromatin (Vaquero *et al.* 2004). It is possible that SIRT1 could influence pre-RC assembly via histone deacetylation and modulates S-phase progression. Furthermore, components and regulators of pre-RC can be acetylated. Hbo1 interacts with the human Orc1 (Iizuka and Stillman 1999), and mouse Mcm2 (Burke *et al.* 2001). *Xenopus* Hbo1 also interacts with Orc1, Orc2, Cdt1, and geminin,

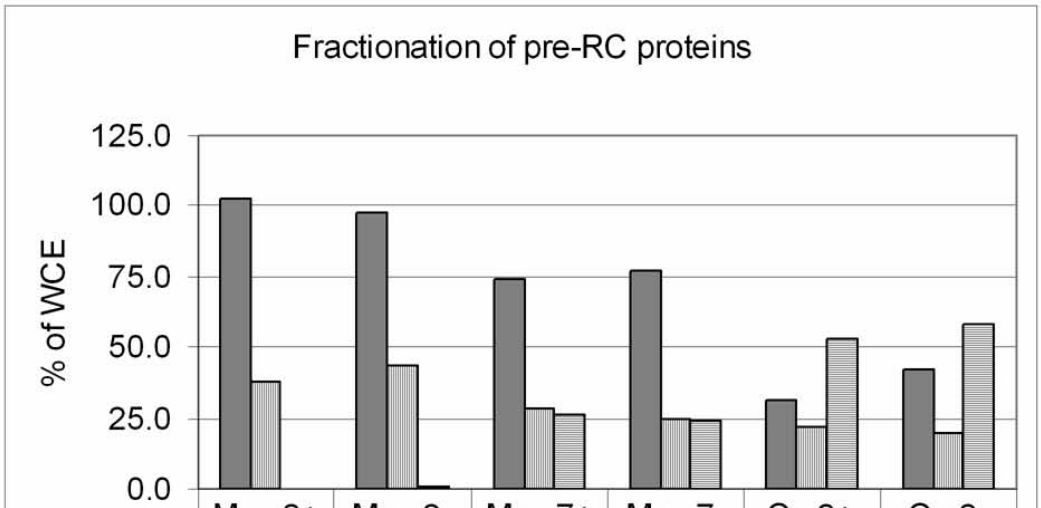
and recombinant human Hbo1 acetylated recombinant human Orc2, Mcm2, Chinese hamster Cdc6, and human geminin *in vitro* (Iizuka *et al.* 2006). Therefore, it is also possible that SIRT1 could influence DNA replication by directly deacetylating these proteins.

Because pre-RC assembly starts soon after Cyclin B/Cdk1 activity drops (Arias and Walter 2007), it is possible that SIRT1 retains phosphorylated residues conferred by Cyclin B/Cdk1. This could influence SIRT1 function in the context of pre-RC assembly, an effect which could persist through S-phase. This model could explain why Sirt1-deficient ES cells have a slower growth rate but are more abundant in S-phase, and why the transfection of FLAG-SIRT1 rescues this effect (**Figs. 18, 19**). It is possible that the SIRT1-AA mutant had a dominant effect on S-phase because it could not be regulated properly by Cyclin B/Cdk1 due to the loss of two target residues.

To test the above idea, I aimed to determine if the presence of SIRT1 affects the distribution of pre-RC proteins in cytoplasm, nucleoplasm, and chromatin fraction. I fractionated proteins from *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ ES cell lysates following the protocol described by Mendez *et al.* (Mendez and Stillman 2000), and analyzed the localization of Orc2, Mcm2, and Mcm7 (**Fig. 27**). I did not detect a difference in the distribution of these pre-RC proteins between wild-type and *Sirt1*-null ES cells. Therefore, it is unlikely that SIRT1 modulates cell proliferation by regulating Pre-RC assembly.



b.



	Mcm2+	Mcm2-	Mcm7+	Mcm7-	Orc2+	Orc2-
S2	102.7	97.1	73.9	76.7	31.3	41.9
S3	38.0	43.5	28.5	24.6	21.9	19.4
P3	-0.4	0.5	26.2	23.6	53.1	58.1

Figure 27. Localization of pre-RC proteins are not affected by the absence of Sirt1 in unsynchronized murine ES cells

a. Fractionation and western blot analysis of Orc2, Mcm2, and Mcm7 proteins. WCE (whole cell extract), S2 (cytoplasmic fraction), S3 (nucleoplasmic fraction), and P3 (chromatin fraction).

b. Densitometric analysis of western blots in shown in **a.** There is no change in the distribution of proteins between *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ ES cells. The Sirt1 band seen on WCE of *Sirt1*$^{-/-}$ ES cells is smaller than full-length Sirt1 and is a non-specific signal.

4.9 Conclusions

The NAD^+-dependent deacetylase SIRT1 is implicated in longevity conferred by calorie restriction. SIRT1 influences survival and health of cells by modulating various processes via its substrates. I found that SIRT1 protein levels correlate with mitotic activity of cells both *in vitro* and *in vivo*. I showed that SIRT1 is phosphorylated at 13 residues, that cyclin and Cdk motifs are conserved among different species, and that Thr530 and Ser540 of SIRT1 are substrates for Cyclin B/Cdk1. Loss of SIRT1 phosphorylation correlates with decrease in the deacetylase activity, and a loss of function mutation for T530 and S540 (AA mutation), two substrates for Cyclin B/Cdk1, eliminates the rescue effect on slow growth and the increase in the fraction of S-phase cells in *Sirt1*$^{-/-}$ ES cells. Therefore, SIRT1 is modulated by the mechanism that controls proliferation of cells, while simultaneously modulating the cell proliferation process itself. SIRT1 is possibly a substrate for many more kinases, and its phosphorylation may regulate its enzyme activity, interaction with other proteins, localization, and turnover.

4.10 Acknowledgement

I thank Dr. Bernhard Maier (Department of Neuroscience/Endocrinology), Dr. Wladek Minor (Department of Molecular Physiology and Biophysics), Dr. Maksymilian Chruszcz (Department of Molecular Physiology and Biophysics), Katarzyna D. Koclega (Department of Molecular Physiology and Biophysics), Dr. Michal Sabat (Department of Chemistry), Dr. Mario Geysen (Department of Chemistry), and Dr. Cyrille Gineste (Department of Chemistry) for their contributions in the investigation for 3D structure of SIRT1.

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