Graduate Theses and Dissertations   Graduate School

2010

A potential tumor suppressive role of SIRT1 in cancer

Neha Kabra
University of South Florida

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the American Studies Commons

A Potential Tumor Suppressive Role of SIRT1 in Cancer

by

Neha Kabra

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Arts and Sciences
University of South Florida

Major Professor: Jiandong Chen, Ph.D.
Alvaro Monteiro, Ph.D.
Kenneth Wright, Ph.D.
Douglas Cress, Ph.D.

Date of Approval:
March 4, 2010

Keywords: Histone deacetylase, Sir2, EX-527, cell proliferation, colon cancer

Acknowledgements

I would like to thank my mentor, Dr. Jiandong Chen, for guiding me throughout my doctoral work. I will always be grateful for the time and effort he has spent training me. I have been very fortunate to be a part of his lab as a graduate student. I would also like to thank my committee members Dr. Kenneth Wright, Dr. Douglas Cress and Dr. Alvaro Monteiro for their invaluable suggestions and support. I would like to thank Dr. James Manfredi for taking time to be my Outside Chairperson for the dissertation defense. I also extend thanks to my friends and lab mates Lihong Chen, Brittany Cross, Zhenyu Li, Qian Cheng, Hong Zheng, Baozong Li, Xiaolong Li, Cynthia Lebron and Daniele Gilkes. I would like to thank Cathy Gaffney for efficiently coordinating the dissertation defense and graduation process. I cannot thank the Cancer Biology Program enough for not only did they give me the opportunity to study at one of the best Cancer Research Institutes in the nation but also because it is through this program that I met my class mate, best friend and husband Nicholas Woods. I am extremely thankful to my parents, Rita and Suresh Kabra who have always motivated and encouraged me and supported me in my decision to come to this country to earn my Ph.D. I am also very fortunate to have a wonderful set of parents-in-law, Judy and David Woods, who have loved me as their daughter since the day I met them. I specially want to thank my husband, Nicholas, for being a constant source of love and support and for always believing in me. I could not have done this without him. Finally, I want to thank my 16-month old son, Nikhil, who has been a shining source of happiness and laughter at the end of each day.

Table of Contents

List of Figures

List of Abbreviations

APC	Adenomatous Polyposis Coli
ATG	Autophagy-related
CBP	CREB-binding protein
CDK	Cyclin-dependent kinases
ChIP	Chromatin Immunoprecipitation
CK-2	Casein Kinase 2
CR	Calorie Restriction
CREB	cAMP response element binding protein
DMSO	Dimethyl Sulfoxide
E2F1	E2F transcription Factor 1
FHL2	Four and a half LIM domains protein 2
FOXO	Forkhead box O
GFP	Green Fluorescent Protein
HAT	Histone Acetyltransferase
HDAC	Histone deacetylase
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
NAD+	Nicotinamide adenine dinucleotide
NES	Nuclear export signal
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear Localization Signal

PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	PPAR gamma coactivator-1 alpha
pRB	Phosphorylated Retinoblastoma protein
RB	Retinoblastoma
rDNA	ribosomal DNA
ROS	Reactive Oxygen Species
shRNA	short hairpin RNA
Sir2	Silent Information Regulator 2
SUMO-1	Small ubiquitin-related modifier 1
UV	Ultraviolet light

A Potential Tumor Suppressive Role of SIRT1 in Cancer

Neha Kabra

ABSTRACT

The NAD-dependent deacetylase SIRT1 regulates several factors involved in stress response and cell survival but its function in cancer is largely unknown. Research suggests that SIRT1 influences several transcription factors and molecules that are important components of pathways often deregulated in cancer. Our experiments have shown that SIRT1 knock down by short hairpin RNA accelerates tumor xenograft formation by HCT116 colon cancer cells, whereas SIRT1 overexpression inhibits tumor formation. We have also found that, pharmacological inhibition of SIRT1 stimulates cell proliferation under conditions of growth factor deprivation suggesting a tumor suppressive function of SIRT1. Paradoxically, SIRT1 inhibition sensitizes the same cells to apoptosis by chemotherapeutic drugs. Immunohistochemical staining of a colon tumor microarray revealed high SIRT1 expression levels in normal colon mucosa and benign adenomas. SIRT1 overexpression was observed in nearly 25% of stage I/II/III colorectal adenocarcinomas but rarely found in advanced stage IV tumors. Furthermore, about 30% of carcinomas showed lower than normal SIRT1 expression. These results suggest a pleiotropic effect of SIRT1 in cancer, i.e., anti-proliferative as well as anti-apoptotic. Further experiments along these lines and examination of a larger patient cohort could provide a rationale for the use of SIRT1 activators and inhibitors in the prevention and treatment of cancer.

Chapter One

Introduction

Cancer

The earliest evidence of cancer was discovered in Egypt in 1600 B.C and it was described as a disease that had no cure. Hippocrates, a Greek physician known as the Father of Medicine, used the terms *carcinos* and *carcinoma* to describe the appearance of cancer. These early physicians were able to crudely diagnose cancers but only after the development of refined autopsy methods in the 17th century leading to the advanced understanding of anatomy and physiology, did the origin and progression of cancer become clear. We now find ourselves in the fortunate position of understanding a portion of the molecular mechanisms that regulate cancer initiation and progression. This knowledge has led to the development of modern cancer therapies that effectively treat many cancers. However, treatment options remain limited and deaths attributed to cancer are still a major cause of mortality throughout the world. To fully combat cancer, continued research into the molecular causes and novel therapies must be completed. A task which seems to become more tangible with each passing day as new technologies for this research becomes available.

Cancer is the second leading cause of death in economically developed countries (following heart diseases). According to the statistics obtained by the U.S National Cancer Institutes, 1 in 2 men in the United States have a lifetime risk of developing cancer while this risk is 1 in 3 for women. Lung cancer has the highest mortality rate in both men and women followed by breast cancer in women and prostate cancer in men (American Cancer Society 2009). These statistics emphasize the need for continued development and progress in the field of cancer research.

Carcinogenesis

Carcinogenesis can be defined as the creation or production of cancer. While cells in a body undergo a tightly regulated cycle of generation, division and death, cancer cells typically evade cell death and are capable of constant multiplication and expansion. They develop traits which are commonly identified as the hallmarks of cancer, namely, self-sufficiency in growth signals and insensitivity to growth-inhibitory signals, evasion of apoptosis, sustained replicative potential and angiogenesis as well as tissue invasion and metastasis (Hanahan and Weinberg, 2000). It has been shown that cancer arises from a single unregulated and genetically unstable cell which divides to form a population of its clones. These clones undergo expansion and accumulate genetic mutations providing a platform for Darwinian selection leading to neoplastic progression of cancer cells best suited for the microenvironment in which they exist (Barrett et al., 1999; Nowell, 1976). In most cases, cellular transformation is a result of activation of oncogenes or suppression of tumor suppressor genes. Cellular oncogenes, also called proto-oncogenes, are normal genes required for important functions in the cell. These genes however, can be transformed into oncogenes by retro-viruses resulting in abnormal cellular proliferation (Cooper, 1982; Haber and Stewart, 1985). On the other hand, tumor suppressor genes or anti-oncogenes limit cellular transformation. These genes encode proteins that inhibit cell cycle progression, promote DNA damage repair and bring about cell death in the event of mutations or stress (Comings, 1973; Sherr, 2004). Knudson's 'two-hit hypothesis' holds true for most tumor suppressor genes wherein two mutational events are required for carcinogenesis. Both alleles of the tumor suppressor gene are required to be lost, mutated or inactivated for manifestation of cancer (Knudson, 1971).

Carcinogenesis is a multistage process that develops through three phases: Initiation, promotion and progression (Pitot and Dragan, 1991) Initiation involves an irreversible change in the cell which is generally an insult to the DNA of the cell. Chemicals such as aromatic

hydrocarbons, radiation (ionizing and ultraviolet) or biological agents such as retroviruses can act as carcinogens to initiate cancer. These carcinogens can cause multiple mutations in the DNA of the cells such that the DNA repair machinery is impaired. As a result, cell cycle check-points are deregulated and the cell divides and proliferates despite the mutations. Tumor Promotion involves the proliferation and expansion of the mutant and genetically unstable cell and accumulation of further mutations with each round of cell division such that the resulting population of cells is capable of surviving in normally unsuitable cellular environments (Cahill et al., 1999). The Progression step comprises of tumor cells that have attained malignant properties, invasiveness and metastatic capabilities. Of note is the fact that mutations that occur during the process of carcinogenesis do not just involve the genetic alteration (deletion, translocation, point mutation, duplication or amplification) of oncogenes or tumor suppressor genes, but can also be epigenetic changes such as modifications of gene promoters by acetylation/ deacetylation or methylation/demethylation (Feinberg et al., 2002; Feinberg and Tycko, 2004). Considering the complexity of cancer, it is of utmost importance to investigate the genes that are involved in its manifestation and the molecular mechanisms which explain their deregulation. Only then can we hope to find the cure.

Tumor Promotion and Tumor Suppression

As mentioned above, oncogenes and tumor suppressor genes play a major role in tumorigenesis. Proto-oncogenes undergo mutation and activation to behave as oncogenes that facilitate tumor formation. Mutations in proto-oncogenes can cause overexpression of its protein product due to deregulation of transcription or gene amplification. Mutations can also be such that the protein product has increased stability or increased activity. Posttranslational modifications of the protein such as phosphorylation can contribute to its stability and constitutive expression which may lead to enhanced cell cycle progression and proliferation. Alterations in tissue specific expression profiles can also cause the gene to behave as an

oncogene. Some examples of oncogenic protein products include RAS, SRC, MYC. RAS is a small GTPase switch that is active when bound to GTP and inactive when bound to GDP (Milburn et al., 1990). RAS regulates signal transduction of several pathways, the most extensively studied being the Mitogen Activated Protein Kinase (MAPK) pathway, which affects cellular processes involved in transformation such as cell proliferation, migration, adhesion, apoptosis, etc. Overexpression of a constitutively active mutant RAS deregulates the signal transduction pathways and promotes invasion and metastasis. Thus, activating RAS mutations are found in several types of cancers (Bos, 1989).

On the other hand, tumor suppressor genes function as anti-oncogenes. Tumor suppressor genes function to repress cell cycle progression and cell division. Upon DNA damage, they facilitate repair but initiate apoptosis upon excessive damage. RB and p53 are two prominent tumor suppressors. They commonly exhibit inactivating mutations and LOH in the manifestation of most cancers (Knudson, 1971). p53 is a transcription factor encoded by the *TP53* gene (Lane, 1992; Lane and Crawford, 1979; Matlashewski et al., 1984). It is maintained at low levels in the cell but upon DNA damage or stress, it gets activated to transcriptionally induce a variety of genes involved in cell cycle check-points or cell death such as *WAF1/CIP1* and *BAX* (Giono and Manfredi, 2006; Miyashita and Reed, 1995; Waldman et al., 1995). RB is a cell cycle inhibiting protein that is modified posttranslationally to regulate its activation. In its hypophosphorylated state (between M to G1), RB is considered active as it efficiently binds to E2F1/DP1 and inhibits their transcriptional function (Helin et al., 1992). As the cell transitions from G1 to S phase, RB is hyperphosphorylated by cyclins and CDK proteins such that it is inactivated and dissociates from the E2F1/DP1 complex thereby allowing transcription of genes that have functional roles in cell cycle progression and division. RB remains hyperphosphorylated throughout S, G2 and M phases (Fattaey et al., 1993). It has also been shown that Rb-E2F1 complex recruits Histone

Deacetylases (HDACs) to the promoters of E2F1 target genes to silence their transcription (Lai et al., 1999).

The details and aspects of tumor promotion and suppression remain elusive and much research is needed in this field. With advances in biological research, new genes are being constantly discovered and analyzed for their potential role in tumorigenesis.

Histone Deacetylases

Histone deacetylases, HDACs, are enzymes that can remove the acetyl moiety from the epsilon amino group of lysine residues on histones. Their function is opposite to that of Histone acetyltransferases (HATs) that transfer acetyl group to the lysine residues. Histones are positively charged proteins that bind tightly to the negatively charged DNA and promote DNA condensation and supercoiling. Acetylation of lysine residues allows for neutralization of the positively charged histones such that the DNA-histone interaction is partially relaxed. This relaxed feature marks an actively transcribing region where transcription factors and other proteins can bind to the DNA. Contrary to HATs, HDACs increase the positive charge on histones by removing the acetyl group from the lysine residues thereby facilitating tighter interaction of histones and DNA and preventing transcription. Histone-DNA and Histone-protein interactions are generally influenced by a combination of histone modifications such as methylation, phosphorylation, ubiquitination and acetylation that together constitute the 'Histone Code' (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Acetylated Lys9 on histone H3; acetylated Lys16 on histone H4 and methylated lys4 on histone H4 together are associated with transcriptionally active DNA. In the late 90s HATs were found to acetylate a few non-histone proteins suggesting that HATs and HDACs were not exclusive to histone modification (Gu and Roeder, 1997). At present, a number of transcription factors are known to be regulated by HDACs and HATs and their importance in cancer is becoming more evident each day.

Classification and Function of Histone Deacetylases

Based on their homology to yeast histone deacetylases, human histone deacetylases have been classified into four groups: Class I which includes HDAC1, 2, 3 and 8 which are similar to yeast RPD3; Class II which includes HDAC4, 5, 6, 7, 9 and 10 which are similar to yeast HDA1; Class III which are also called sirtuins have seven family members (SIRT1-7) and are homologous to yeast Sir2 gene and Class IV which includes HDAC11 that has features similar to Class I and Class II (Glozak and Seto, 2007; Gregoretti et al., 2004). Class I and II share sequence similarity and are called the classical HDAC enzymes (Figure 1). They require Zn^{2+} for their catalytic activity. Sirtuins do not share any sequence similarity with the classical HDACs and require NAD+ to carry out the enzymatic reaction.

Class I and II Histone Deacetylases.
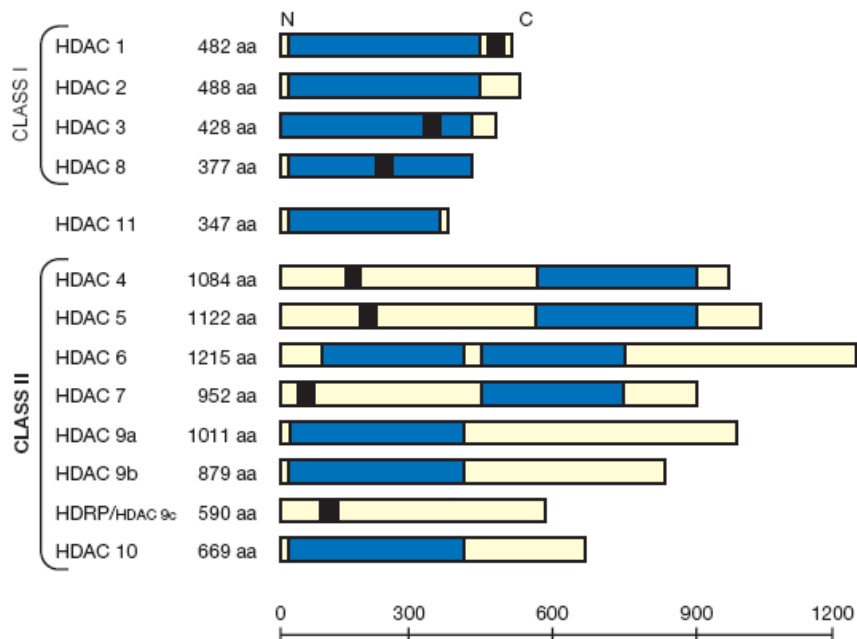
Fig1: **Classification of HDAC Class I and II**. The Bars depict the length of the protein. The catalytic domain is shown in blue and NLS is shown in black (de Ruijter et al., 2003).

Class I Histone Deacetylases. These include HDAC1, 2, 3 and 8. Members of this class contain an N-terminal deacetylase domain and a C-terminal tail.

HDAC1 was the first histone deacetylase to be identified and subsequently characterized (Taunton et al., 1996). It has an N-terminal deacetylase domain and a C-terminal tail which contains tandem CK-2 phophorylation sites as well as a sumoylation motif (Sengupta and Seto, 2004). Phosphorylation of HDAC1 has been shown to enhance its deacetylase function and mutation of these phosphorylation sites impairs its enzymatic activity as well as inhibits its ability to form complexes with other co-repressors (Pflum et al., 2001). HDAC1 and 2 are usually a part of protein complexes that are recruited to the DNA by DNA binding proteins and these protein complexes enhance the enzymatic activity of HDACs. Three such complexes have been identified, namely, Sin3 (Grzenda et al., 2009), NuRD (Denslow and Wade, 2007) and CoREST (Grozinger and Schreiber, 2002; You et al., 2001) which mediate chromatin modification and transcription repression by deacetylation and methylation and hence play a significant role in maintaining cell cycle progression, genomic stability and homeostasis. Besides these multiprotein complexes, HDAC1 has been shown to associate with retinoblastoma protein (RB) and inhibit cell cycle progression (Luo et al., 1998). DNMT1 or DNA Methyltransferase1 has also been shown to associate with HDAC1, RB and E2F1 in a complex that represses transcription from E2F1 target genes (Robertson et al., 2000). However, disruption of both HDAC1 alleles results in embryonic lethality due to proliferation defects. Cyclin-dependent kinase inhibitors p21 and p27 are upregulated in HDAC1-deficient embryos resulting in reduced cellular proliferation and histones H3 and H4 are hyperacetylated thereby causing changes in other histone modifications (Lagger et al., 2002). HDAC1 has also been shown to be upregulated in prostate cancer with concomitant decrease in p21 levels thereby promoting cellular proliferation (Halkidou et al., 2004). HDAC1 itself can be regulated by posttranslational modifications such as sumoylation by SUMO-1 and

7

according to one study, this modification can enhance the transcriptional repression function of HDAC1 (David et al., 2002).

HDAC2 bears a large sequence similarity with HDAC1 and is present in many protein complexes with HDAC1. It has beenfound to interact with YY1 and promote transcriptional repression (Yang et al., 1996). It has also been shown to interact with DNA Methyltransferase DNMT1 to bring about transcriptional repression (Rountree et al., 2000). HDAC2 can negatively regulate the transcriptional function of NF-κB via association with HDAC1 (Ashburner et al., 2001). It is also overexpressed in the polyp stage of colorectal carcinoma (Huang et al., 2005) and this elevated expression is induced upon loss of the Adenomatous Polyposis Coli (*APC*) gene (Zhu et al., 2004). Similar to HDAC1, decrease in HDAC2 levels correlate with increased p21 levels and apoptosis (Huang et al., 2005).

HDAC3 was identified and cloned based on sequence similarities to HDAC1 and 2 (Yang et al., 1997). Similar to HDAC2, it bind to YY1 and acts as a transcriptional repressor. However, unlike HDAC1 and 2 which are localized in the nucleus, HDAC3 contains both nuclear import and export sequences and therefore, can be localized in the nucleus as well as the cytoplasm (Takami and Nakayama, 2000; Yang et al., 2002). HDAC3 has also been shown to form a stable complex with nuclear hormone receptor corepressor, NCoR. The HDAC3- NCoR interaction enhances the repressive activity of NCoR and the deacetylase activity of HDAC3 by promoting a more favorable conformation of the latter (Wen et al., 2000).

HDAC8, the fourth member of Class I HDACs, was cloned and characterized based on sequence similarity to the other Class I histone deacetylases. It is sub-nuclear in localization and the gene encoding this HDAC is located on the X-chromosome (Buggy et al., 2000; Hu et al., 2000; Van den Wyngaert et al., 2000). HDAC8 is phosphorylated by protein kinase A (PKA) and this phosphorylation inhibits the deacetylase activity of HDAC8 thereby resulting in hyperacetylated histones H3 and H4 (Lee et al., 2004; Yang and Seto, 2008). CREB, which is an important activator of genes involved in metabolism and survival, is activated upon

phosphorylation. It has been found that HDAC8 can form a complex with CREB and PP1, a dephosphatase enzyme to bring about inactivation of CREB transcriptional function. Hence, HDAC plays an important role in transcription regulation (Gao et al., 2009). Knock down of HDAC8 by siRNA inhibits growth of lung, colon and cervical cancer cell lines suggesting that this histone deacetylase contributes to tumor cell proliferation (Vannini et al., 2004).

Class II Histone Deacetylases. These are homologous to yeast HDA1 or Histone Deacetylase1 and are further classified into two groups: Class IIa which includes HDAC4, 5, 7 and 9 and Class IIb which includes HDAC6 and 10. The Class IIa HDACs have an extended N-terminus which contains sites for 14-3-3 and myocyte enhancer factor2 (MEF2) binding. The MEF2 binding motif is conserved from *C. elegans* to mammals (Yang and Gregoire, 2005).

HDAC4 is the founding member of the Class II Histone deacetylases. Unlike the Class I HDACs which are primarily nuclear, HDAC4 can shuttle between the nucleus and cytoplasm through active nuclear export. In the nucleus, HDAC4 can associate with MEF2A and repress its transcriptional activation thereby repressing skeletal myogenesis (Miska et al., 1999). Differential localization of HDAC4 may be a way to regulate the deacetylase effect of this enzyme. HDAC4 has an N-terminal nuclear import sequence and C-terminal nuclear export motif. It binds to 14-3-3 which facilitates its cytoplasmic localization (Wang et al., 2000; Wang and Yang, 2001). Furthermore, ERK1/2 have been shown to associate with HDAC4 in a complex and subsequently phosphorylate HDAC4 (Zhou et al., 2000b) thereby placing this histone deacetylase in the MAPK pathway. Expression of oncogenic RAS is associated with increased nuclear HDAC4 and decreased MEF2-mediated transcription.

HDAC5 was identified as a deacetylase that was involved in chromatin modeling during cell differentiation (Verdel and Khochbin, 1999). Similar to HDAC4, HDAC5 can shuttle between nucleus and cytoplasm. It interacts with 14-3-3 and this interaction facilitates its cytoplasmic

localization which is thought to regulate its enzymatic effect (Grozinger and Schreiber, 2000). HDAC5 can also interact with MEF2 and inhibit its transcriptional function (Lemercier et al., 2000). However, this interaction and repression of MEF2 activity is independent of HDAC5 deacetylase activity. The N-terminal non-deacetylase domain of HDAC5 is responsible for this repression despite the presence of a fully functional deacetylase domain. It is hypothesized that the repressive function of HDAC5 on MEF2 may be through the recruitment of other HDACs such as HDAC4 and HDAC3 (Grozinger et al., 1999)

HDAC7 was identified and characterized based on sequence similarities with HDAC4 and 5 (Fischle et al., 2001). It has a deacetylase domain in the C-terminal half and an NLS sequence in the N-terminal half of the protein. The enzymatic activity of HDAC7 is dependent on its interaction with HDAC3. HDAC7 does not directly bind to HDAC3 but the two HDACs are recruited by the corepressors SMRT and N-CoR (Fischle et al., 2001). HDAC7 can also shuttle between nucleus and cytoplasm but only nuclear HDAC7 is associated with enzymatic activity (Fischle et al., 2001). Calmodulin Kinase I or CAMK I has been shown to phosphorylate HDAC7. Phosphorylated HDAC7 interacts with 14-3-3 which facilitates its cytoplasmic localization and stabilization (Li et al., 2004).

HADC9 was cloned and characterized based on sequence similarity to HDAC4 and the other Class IIa histone deacetylases (Zhou et al., 2001). It has splice variants 9a, 9b and 9c (or Histone deacetylase-related protein, HDRP). HDAC9a and 9b lack the nuclear localization sequence and have the deacetylase domain in the N-terminal half of the protein while 9c or HDRP (also called MITR, MEF2-interacting transcription repressor) lacks the deacetylase domain altogether but retains the NLS sequence (de Ruijter et al., 2003). HDRP can form a complex with HDAC1 and HDAC3 to compensate for the lack of its deacetylase domain (Zhou et al., 2000a). It can associate with corepressor CtBP and the other HDACs to repress MEF2 activity (Zhang et al., 2001). Interestingly, HDAC9 gene promoter contains sites for MEF2 binding and it has been found that MEF2 can transcriptionally activate HDAC9 during muscle

cell differentiation thereby suggesting the existence of a negative feedback loop between HDAC9 and MEF2 (Haberland et al., 2007).

Class IIb consists of HDAC6 and HDAC10. These two HDACs resemble each other but show lesser similarity with the Class IIa HDACs.

HDAC6 is unique because it contains two tandem catalytic domains (Grozinger et al., 1999; Verdel and Khochbin, 1999). It was also identified as the first HDAC that was actively maintained in the cytoplasm but was capable of nuclear localization (Verdel et al., 2000). A strong nuclear export signal is located N-terminal to the first catalytic domain which prevents the accumulation of this protein in the nucleus. This HDAC also contains a tetradecapeptide repeat domain C-terminal to the second catalytic domain. This set of eight repeats, named SE14, display acetyl microtubule targeting ability as well as form a unique structure which is required for leptomycin-B –resistant cytoplasmic localization of HDAC6 (Bertos et al., 2004). The presence of NES and SE14 ensure the cytoplasmic anchorage of HDAC6. C-terminal to the SE14 repeats is another domain unique to HDAC6. This is a cysteine and histidine-rich domain named ZnF-UBP which is present in several ubiquitin-specific proteases. This domain allows HDAC6 to bind to mono- and poly-ubiquitin chains (Boyault et al., 2006; Seigneurin-Berny et al., 2001). The high-affinity binding of HDAC6 to ubiquitin interferes with the effective proteasomal degradation of the ubiquitinated proteins. p97/VCP can bind to HDAC6 and dissociate it from the ubiquitin complex thereby allowing the ubiquitinated proteins to be processed further (Boyault et al., 2006). While there is no evidence of histone deacetylation by HDAC6 *in vivo,* a few non-histone substrates of HDAC6 have been discovered. HDAC6 has been shown to deacetylate alpha-tubulin in assembled microtubules (Hubbert et al., 2002; Matsuyama et al., 2002). Since proteins like kinesin-1 require acetylated alpha-tubulin to transport proteins such as JIP1 (Reed et al., 2006), HDAC6 interferes with this transport by destabilizing the microtubules. Besides alpha-tubulin, HDAC6 deacetylates the molecular chaperone Hsp90 and facilitates the assembly of protein complexes involved in cell signaling

(Kovacs et al., 2005). In the absence of HDAC6, Hsp90 becomes hyperacetylated,

dissociates from its co-chaperone and loses its ability to form the chaperone complex.

Despite its cytoplasmic localization, HDAC6 can be localized to the nucleus in certain cases

and act as deacetylase on nuclear targets. It is recruited by RUNX2 from the cytoplasm to the

promoter of *WAF1/CIP1* to encode p21 protein in differentiating osteobalsts (Westendorf et

al., 2002) thereby regulating tissue-specific gene expression.

HDAC10 was identified based on sequence similarity with HDAC6 (Fischer et al., 2002;

Guardiola and Yao, 2002). It contains two catalytic domains as well but the C-terminal

catalytic domain is non-functional. HDAC10 has been found to be localized in the nucleus as

well as the cytoplasm and has alternatively spliced variants (Fischer et al., 2002; Guardiola

and Yao, 2002; Kao et al., 2002). It has also been found to interact with HDAC2 and SMRT

complexes suggesting a role in promotion of transcriptional repression (Fischer et al., 2002).

Class III Histone Deacetylases (Sirtuins). Sirtuins are homologous to the proteins encoded

by the yeast SIR genes and this class of histone deacetylases is significantly different from

Class I and II in their structure, enzymatic activity, localization and function (Figure 2).
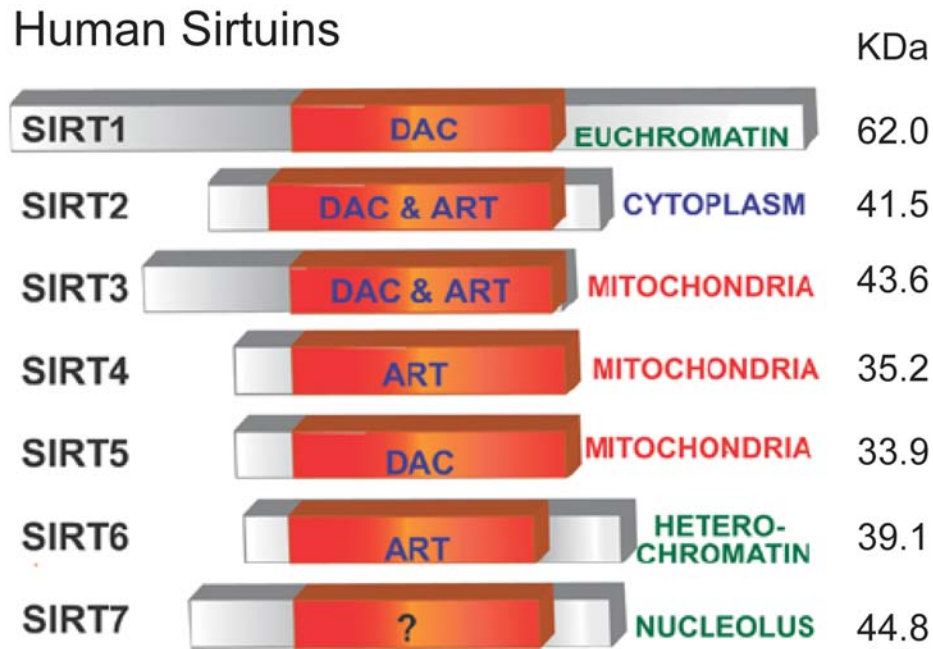
Fig 2: Class III HDACs (Sirtuins). Mammals have seven sirtuins, SIRT1–7. All have an NAD+-dependent catalytic core domain that may act preferentially as a mono-ADP-ribosyl transferase (ART) and/or NAD+-dependent deacetylase (DAC). Additional N-terminal and/or C-terminal sequences of variable length may flank this core domain. The seven sirtuins show different cellular localization (Michan and Sinclair, 2007).

The yeast Sir2 gene was discovered as a suppressor of recombination between copies of tandem repeated rDNA (Gottlieb and Esposito, 1989). Sir2 was also found to silence genes near the telomeres in *S. cerevisiae* (Aparicio et al., 1991). Later, it was elucidated that the silencing of the mating-type loci and telomeres was associated with hypoacetylation of the histones at the epsilon-amino group of N-terminal lysine residues and that overexpression of Sir2 was responsible for histone deacetylation *in vivo* (Braunstein et al., 1993). The discovery of Sir2 homologs in various organisms, from bacteria to humans, suggested that it belongs to an evolutionarily conserved family of genes.

Studies with bacterial homolog CobB indicated that Sir2 may possess NAD+ -dependent mono-ADP-ribosyltransferase activity (Tsang and Escalante-Semerena, 1998). By 1999, five

human cDNA homologous to yeast Sir2 had been discovered and it was experimentally established that these sirtuins can metabolize NAD+ and function via mono-ADP-ribosylation of proteins (Frye, 1999). It was also shown that Sir2 catalyzed the deacetylation reaction of an acetylated lysine residue by utilizing NAD+ and producing nicotinamide, deacetylated lysine and 1-O-acetyl-ADP-ribose as the end products (Tanner et al., 2000). Therefore, Sir2 coupled two enzymatic reactions; deacetylation and NAD+ metabolism. In this regard, members of Sir2 family, sirtuins, differ from the other two classes of HDACs. The other classes of HDACs require Zn^{2+} for their catalytic function while sirtuins require NAD+.

A number of studies have suggested a role for sirtuins in aging. In yeast, presence of ERCs (Extra-chromosomal Circles) accelerates aging (Sinclair and Guarente, 1997). These ERCs are a result of recombination between rDNA repeats. Amplification of ERCs titrates the replication machinery from genomic DNA as a result of which accumulating ERCs shorten the life-span of yeast cells (Sinclair and Guarente, 1997). It was found that Sir2 could induce transcriptional silencing at the rDNA locus (Fritze et al., 1997) and introduction of an extra copy of Sir2 could increase the yeast lifespan up to 30% by suppressing rDNA recombination (Kaeberlein et al., 1999). Eventually, Sir2-mediated increase in life-span was also reported in *C. elegans* (Tissenbaum and Guarente, 2001) followed by *Drosophila* (Rogina and Helfand, 2004). Increased life-span upon calorie restriction (CR) has been demonstrated in a variety of organisms from yeast to mammals. Sir2 was found to play a role in increasing life-span upon CR in yeast. In Sir2 deleted genotypes, CR did not increase life-span in yeast or *Drosophila* suggesting the importance of Sir2 in CR-mediated longevity (Lin et al., 2000; Rogina and Helfand, 2004). It was proposed that CR promoted respiration instead of fermentation which increased the NAD+/NADH ratio by decreasing NADH levels thereby stimulating the activity of Sir2 (Lin et al., 2002). However, recent studies have shown that calorie restriction can reduce the frequency of rDNA recombination and increase yeast life-span independently of Sir2-

mediated rDNA silencing (Riesen and Morgan, 2009). The importance of human sirtuins in mediating CR-induced longevity is currently being evaluated.

The mammalian sirtuin family consists of seven members from SIRT1-7. Each sirtuin has a conserved 275 amino acid catalytic core domain and unique N and C-terminal sequences as well as different cellular localizations (Figure 2). The biological functions of these sirtuins are still being elucidated since this is a relatively new field of research.

SIRT1: The first member of human sirtuins. It is the closest homolog of yeast Sir2 gene (Frye, 2000) and perhaps the most investigated of all sirtuins. It is localized in the nucleus and functions as an NAD+ -dependent histone as well as protein deacetylase. Knockout experiments in mice have shown that Sirt1 is important for embryonic development. It was found that the severity of the phenotype of Sirt1 null mice depended on the genetic background of the mice but in most cases, the loss of Sirt1 was embryonic lethal. However, a very small proportion of Sirt1 null mice were born viable but failed to survive more than a few months beyond birth (Cheng et al., 2003; McBurney et al., 2003). These viable mice had obvious phenotypic abnormalities including smaller size, sterility and eye and heart development defects.

SIRT1 has been shown to deacetylate histone H3 lys9, histone H4 lys16 and histone H1 lys26 (Vaquero et al., 2004) thereby facilitating chromatin condensation and compaction leading to transcription repression. Some of the earliest studies on SIRT1 as a protein deacetylase were done with the transcription factor p53 where it was shown that SIRT1 could deacetylate and inhibit the transcriptional function of p53 (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001). Since p53 is a well known tumor suppressor, its inhibition by SIRT1 categorized SIRT1 as an oncogene. Recent research, however, has shown that while SIRT1 deacetylates p53, there is no biological outcome of this deacetylation (Kamel et al., 2006; Solomon et al., 2006). Besides p53, SIRT1 has also been shown to influence several different

biological pathways by deacetylating and regulating various transcription factors such as the forkhead family or FOXO (Motta et al., 2004), Ku70 (Cohen et al., 2004a), NFκB (Yeung et al., 2004), E2F1 (Wang et al., 2006), NBS1 (Yuan and Seto, 2007) to name a few. Importantly, SIRT1 also influences metabolic pathways by regulating key proteins such as PPARγ (Picard et al., 2004) and PGC-1α (Nemoto et al., 2005). By doing so, SIRT1 plays a significant role in mitochondrial biogenesis and metabolism such as fat mobilization upon calorie restriction. SIRT1 has also been shown to play an important role in insulin signaling pathway (Kloting and Bluher, 2005). SIRT1 levels were found to be low in insulin resistant cells and overexpressing SIRT1 improved insulin sensitivity. This aspect of SIRT1 function could prove to be useful in managing type 2 diabetes (Sun et al., 2007). The function and regulation of SIRT1 has been described in detail later in this Introduction section.

SIRT2: Unlike SIRT1, SIRT2 is cytoplasmic in its localization (Perrod et al., 2001). It possesses mono-ADP-ribosyl transferase as well as NAD+ dependent deacetylase activity. It has been shown to deacetylate α-tubulin thereby affecting the biological function of microtubules in the cell. SIRT2 can associate with HDAC6 in a complex or work independently to deacetylate tubulin (North et al., 2003) This enhances microtubule stability and cell motility. SIRT2 also interacts with the homeobox transcription factor HOXA10 thereby suggesting a role in mammalian development although the exact effect of this interaction on the function of either of these proteins is not clear (Bae et al., 2004). It has also been shown that SIRT2 overexpression leads to a prolonged mitotic phase of the cell cycle and decrease in SIRT2 protein levels is essential for mitotic exit in the cell cycle (Dryden et al., 2003). However, the molecular mechanism by which SIRT2 regulates the cell cycle is not understood very well. According to a recent report, SIRT2 can localize to the nuclei during the G2/M phase of the cell cycle and deacetylate histone H4 lys16 (Vaquero et al., 2006). This is thought to facilitate the condensation of chromosomes during the mitotic phase (Inoue et al.,

2007). Research has also shown that similar to SIRT1, SIRT2 can regulate FOXO3a by deacetylation upon stress and calorie restriction. Deacetylation by SIRT2 promotes DNA binding by FOXO and induces the transcription of FOXO target genes to express proteins such as p27^{kip1}, manganese superoxide dismutase and pro-apoptotic BIM. As a result, SIRT2 is instrumental in decreasing ROS in the cell as well as promoting apoptosis upon stress (Wang et al., 2007). Hence, even though SIRT2 is cytosolic, it retains its deacetylase activity and can regulate important cellular processes.

SIRT3: Similar to SIRT2, SIRT3 exhibits ADP-ribosyl transferase as well as NAD+ dependent deacetylase activity. It localizes to the mitochondria due to the presence of a unique N-terminal mitochondrial localization sequence (Onyango et al., 2002). Further investigation showed that SIRT3 is in fact localized to the inner mitochondrial membrane and its levels are higher in brown adipose tissues as compared to the white adipose tissues. Interestingly, levels of *SIRT3* mRNA increase in response to calorie restriction and environmental temperature stress in brown adipose tissues of rodents suggesting a role in adaptive thermogenesis (Shi et al., 2005). In the mitochondria, SIRT3 promotes the expression of the master metabolic regulator PGC-1α, uncoupling protein UCP1 and ATP synthetase thereby activating mitochondrial genes, respiration and oxygen consumption (Shi et al., 2005). A recent report suggested that SIRT3 is a mitochondrial as well as nuclear sirtuin. It normally resides in the nucleus where it deacetylates histone H4 lys16 and to some extent histone H3 lys9 such that it is capable of inhibiting gene transcription but localizes to the mitochondria upon stress signals such as UV or etoposide. It is hypothesized that the nuclear exit of SIRT3 upon stress may be required for the rapid activation of nuclear genes that are needed to respond to the stress (Scher et al., 2007). Interestingly, one study showed a functional polymorphism of SIRT3 which was associated with longer life-span in humans, thereby making this the only sirtuin directly linked to human longevity (Bellizzi et al., 2005).

SIRT4: SIRT4 is also a mitochondrial localized sirtuin (Michishita et al., 2005). It functions as an ADP-ribosyl transferase instead of a histone deacetylase (Ahuja et al., 2007). Its expression in pancreas has been found to be intensely associated with insulin secretion (Haigis et al., 2006). SIRT4 utilizes NAD+ for ADP ribosylation of glutamate dehydrogenase (GDH), an enzyme that converts glutamate to α-ketoglutarate in the mitochondria of pancreatic β-cells. ADP ribosylation represses the enzymatic activity of GDH thereby inhibiting glutamate metabolism which is required to generate ATP (Haigis et al., 2006). Since ATP promotes insulin secretion, SIRT4 represses the secretion of insulin from pancreatic β cells in response to glutamate.

SIRT5: SIRT5 is a mitochondrial deacetylase (Michishita et al., 2005) and is extensively expressed in a number of tissues (Frye, 1999). Recently, it has been shown that SIRT5 can deacetylate natively acetylated cyctochrome *c* present in the mitochondrial inter-membrane space which can affect its role in respiration or formation of the apoptosome (Schlicker et al., 2008). Besides cytochrome *c*, the limited knowledge of SIRT5 functions also includes its deacetylation of CPS1 (carbamoyl phosphate synthatase 1) which is an important enzyme in the urea cycle for ammonia detoxification (Nakagawa et al., 2009). SIRT5 upregulates CPS1. This is especially important during fasting when elevated NAD+ levels promote amino acid catabolism and increased blood ammonia levels. Therefore, SIRT5 plays an important role in ammonia detoxification.

SIRT6: Similar to SIRT1, SIRT6 is a nuclear enzyme which exhibits ADP-ribosyl transferase activity (Liszt et al., 2005). It is highly associated with heterochromatic regions in the nucleus (Michishita et al., 2005). SIRT6 has been shown to be involved in Base Excision Repair (BER) and its loss leads to age- associated degenerative processes (Mostoslavsky et al.,

2006). Recent reports show that SIRT6 levels increase upon nutrient deprivation and this increase is due to stabilization of the protein. Also, p53 positively regulates the levels of SIRT6 under normal conditions but not under stress conditions (Kanfi et al., 2008b). SIRT6 has been shown to deacetylate histone H3 lys9 at telomeric regions thereby propagating a stabilized chromatin state at the telomeres. Loss of SIRT6 results in telomeric fusion and premature senescence (Michishita et al., 2008). SIRT6 may also contribute to the maintenance of genomic stability by deacetylating H3 lys56 at the telomeres (Michishita et al., 2009; Yang et al., 2009). Moreover, SIRT6 has been shown to interact with the rel A subunit of NFκB and deacetylate histone H3 lys9 at the NFκB target gene promoters resulting in silencing of transcription of genes involved in apoptosis and cellular senescence (Kawahara et al., 2009). The role of SIRT6 as an important regulator of cellular processes is becoming more evident with ongoing research.

SIRT7: The last member of the sirtuin family, SIRT7 is nucleolar in its localization (Frye, 2000; Michishita et al., 2005). It is a relatively newly discovered sirtuin whose biological functions are not completely understood. It has been found that SIRT7 is a component of the RNA polymerase I transcriptional machinery and it stimulates Pol I transcription. The exact substrate of SIRT7 in this pol I complex is not known. Neither ADP-ribosyl transferase nor deacetylase activity of SIRT7 is known. Therefore, it utilizes NAD+ to activate the Pol I machinery by some unique mechanism that is yet to be discovered (Ford et al., 2006).

Class IV Histone Deacetylases (HDAC11). HDAC11 is the only member of this class of histone deacetylases. It was discovered based on sequence similarity to both class I and II HDACs in the core catalytic domain (Gao et al., 2002). It is localized in the nucleus and only expressed in some tissues which suggests that it may have tissue-specific functions. Interestingly, it has been found to be overexpressed in some cancer cell lines and therefore

its role in tumorigenesis is being investigated. It is commonly found in protein complexes that contain HDAC6 (Gao et al., 2002). HDAC11 specifically associates with CDT1 which is a replication licensing factor. It deacetylates CDT1 at lysine residues thereby facilitating ubiquitinylation and degradation of the latter. Thus, HDAC11 regulates the stability of CDT1 (Glozak and Seto, 2009). Recently, a role for HDAC11 in immune activation has been demonstrated. HDAC11 is expressed in Antigen Presenting Cells (APCs) and functions to negatively regulate the expression of IL-10 while inducing inflammatory APCs which can prime naïve T cells and restore responsiveness of tolerant T cells (Villagra et al., 2009). Since HDAC11 is a newly discovered histone deacetylase, a comprehensive list of its biological functions is still in the making.

Pharmacological Inhibitors and Activators of HDACs

Considering the large number of cellular processes that are regulated by histone deacetylases, it is evident that their deregulation will be detrimental to the cell. These HDACs function as 'lysine deacetylases' since they deacetylate histone as well as non-histone proteins (Xu et al., 2007) resulting in alteration of transcription, gene regulation, protein modification, DNA repair and cell division. Deregulation of HDACs can either lead to cellular apoptosis or cellular transformation and it is for the latter that HDAC inhibitors have been extensively investigated. Cellular transformation generally involves the repression of tumor suppressor genes, cell cycle inhibitors and apoptosis inducers and many of these repressive effects are due to recruitment of HDACs. Therefore, HDAC inhibitors are an important aspect of cancer therapy and management.

Research has shown that of the 18 HDACs that have been discovered, none of them are redundant in their biological responsibilities. However, they have a certain degree of similarity in their core domains that allow us to group them into three major classes. As mentioned previously, class I and II HDACs require Zn+ for their enzymatic activity while class III HDACs

20

require NAD+. These common features have allowed for the design of some effective HDAC inhibitors. The exact mechanism of HDACi- induced death of transformed cells is complex and not very well understood but several of these compounds are in clinical trials due to their effectiveness in inhibiting transformed cell proliferation and the relative resistance of normal cells to their toxic effects.

Inhibitors of HDAC Class I and II. Sodium butyrate was the first histone deacetylase inhibitor (HDACi) to be discovered. It was found that tumor cells treated with sodium butyrate exhibited an increase in acetylation of histone H3 and H4 (Boffa et al., 1978; Candido et al., 1978). It was also found that the acetylated DNA was more accessible for attack by DNAse I (Boffa et al., 1978; Vidali et al., 1978). The molecular mechanism of action of this inhibitor is not clearly defined although its effects on several biological pathways have been described. Due to its function as a histone deacetylase inhibitor (Sealy and Chalkley, 1978), this compound has been shown to activate the transcription of p21, a major cyclin/cdk suppressor, thereby leading to cell cycle arrest (Chen et al., 2004) as well as up-regulate the transcription of various genes involved in the pro-apoptotic machinery of the cell (Pajak et al., 2007). Sodium butyrate was followed by the discovery of Trichostatin A (TSA) and Trapoxin. Both agents could effectively inhibit histone deacetylases and induce differentiation and cell cycle arrest (Van Lint et al., 1996; Yoshida et al., 1995). Trapoxin increased the histone H3 and H4 acetylation levels in the *WAF1/CIP1* promoter thereby inducing p21 protein expression. Increase in p21 protein levels resulted in a decrease in RB phosphorylation levels thereby causing G1 and G2 cell cycle arrest in certain cancer cell lines (Sambucetti et al., 1999). Vorinostat (SAHA) is structurally similar to TSA and is a pan inhibitor of Class I and II HDACs (Marks and Breslow, 2007; Richon et al., 1998). The mechanism of action of SAHA is complex and not completely understood. It has been shown that SAHA can bind to its ligands with sufficient strength so as to induce the accumulation of acetylated histones and alter gene

transcription and expression of proteins that are involved in proliferation, migration and apoptosis in transformed cells resulting in cell death (Richon et al., 1998). At the same time, this binding of SAHA to its ligand is rapidly reversible thereby allowing for some deacetylation to occur. This rapid reversibility does not make SAHA the most potent HDACi but strong enough to induce transformed cell death with minimum toxicity to normal cells. SAHA has been used successfully in culture and animal models to inhibit transformed cell growth with negligible toxicity. Normal cells are ten-fold more resistant to SAHA as compared to transformed cells which makes it an attractive candidate for cancer therapy (Ungerstedt et al., 2005). At present, SAHA is being used in clinical trials and has shown promise in the treatment of solid and hematological malignancies with very few side effects (Marks and Breslow, 2007). Besides SAHA, other HDAC inhibitors include Dispeptide, Valproic Acid, PDX101 etc which are effective against Class I and II HDACs and are being tested as potential drugs for clinical trials (Dokmanovic et al., 2007).

Inhibitors of HDAC Class III (Sirtuins). As mentioned earlier, Class III HDACs are unique and different from the other two classes of HDACs. They require NAD+ for their enzymatic activity and deacetylate the substrate along with metabolism of NAD+ to release nicotinamide, O-acetyl ADP-ribose and the deacetylated substrate. The deacetylation reaction involves an enzyme-ADP-ribose intermediate (Landry et al., 2000). Knowledge of the biological roles of sirtuins is not comprehensive and current research is elaborating the list of interacting proteins and pathways for this class of HDACs. Of the sirtuins, SIRT1 is the most extensively investigated HDAC. Its function in connection with cancer, cardiac diseases and neurodegenerative disorders is being extensively studied. Inhibitors of SIRT1 and other sirtuins provide us with an important means to study their role in human diseases. Nicotinamide is the earliest known and most obvious inhibitor of the deacetylase enzymatic reaction. It drives the ADP-ribosylation reaction backward towards the acetyl lysine and NAD+

thereby inhibiting the enzymatic reaction mediated by SIRT1 (Jackson et al., 2003).

Analogues of NAD+ have also been developed such as carba-NAD+ which is a non-hydrolysable competitive inhibitor of SIRT1 (Landry et al., 2000). Splitomicin was discovered as a Sir2 inhibitor in yeast when it was found that this drug could inhibit the silencing of mating type locus, telomeres and rDNA recombination (Bedalov et al., 2001). Next, Sirtinol was discovered by reverse genetics as an inhibitor of yeast and human Sir2 deacetylase activity (Grozinger et al., 2001). It was later shown that Sirtinol could induce cellular senescence in breast cancer and lung cancer cell lines. This senescence was accompanied with impaired activation of MAPK pathways thereby suggesting an anticancer role for this drug (Ota et al., 2006). Cambinol, a SIRT1 and SIRT2 deacetylase inhibitor was identified and found to be competitive with the peptide and not NAD+. It was shown to have anti-tumor activity in BCL6- mediated Burkitt lymphoma. Treatment of Burkitt lymphoma with Cambinol resulted in apoptosis which was associated with hyperacetylation of BCL6 and p53. Since acetylation inactivates BCL6 but activates p53 transcriptional function, Cambinol exhibited antitumor properties (Heltweg et al., 2006). Moreover, Cambinol was successful in inhibiting tumor growth in mouse xenograft experiments.

Based on high-throughput screening, indoles were discovered as potent SIRT1 inhibitors. This class of inhibitors was specific for SIRT1 over any other sirtuin and its mode of action was different from any other inhibitors known so far. These inhibitors bound to the enzyme after the substrate and NAD+ had already been bound and nicotinamide had been released but the release of the deacetylated product and O-acetyl-ADP ribose was inhibited (Napper et al., 2005). Later, the effect of a specific indole EX-527 was investigated in detail and found to be highly specific against SIRT1 in nanomolar amounts. It was found that while inhibition of SIRT1 deacetylase activity by EX-527 promoted an increase in p53 acetylation at lys382, there was no effect on viability or p53 controlled gene expression in cells treated with etoposide (Solomon et al., 2006).

Very recently, another set of SIRT1 and SIRT2 inhibitors were identified based on yeast genetics and biochemical assays called tenovins (Lain et al., 2008). A cell based assay was carried out to identify compounds that could activate p53 and its downstream target genes and tenovins were discovered from the compound library. These tenovins were found to decrease tumor growth in xenograft experiments and capable of inhibiting SIRT1, SIRT2 and to some extent SIRT3 deacetylase activity.

Activators of Sirtuins. Resveratrol, a polyphenol found in grape and grape products, was found to activate yeast Sir2 and human SIRT1. It was found to lower the Michaelis constant of SIRT1 for both the acetylated substrate and NAD+ thereby promoting the deacetylase activity of SIRT1 and increasing cell survival by stimulating SIRT1-dependent deacetylation of p53 (Howitz et al., 2003). It was suggested that resveratrol could mimic calorie restriction conditions and potentially increase longevity in yeast. However, in light of recent research, the role of resveratrol as a SIRT1 activator is controversial specifically because of its ability to promote SIRT1 activity in a single *in vitro* assay but non reproducible effects in cells (Borra et al., 2005; Kaeberlein et al., 2005).

Recently, small molecule activators of SIRT1 were identified that were structurally unrelated to resveratrol and exhibited almost 1,000 fold more potency than resveratrol. These compounds were shown to function by binding to the SIRT1 enzyme-peptide substrate complex at an allosteric site amino-terminal to the catalytic domain and lowering the Michaelis constant for acetylated substrates. Among them, SRT1720 was the compound of choice and it was shown that administration of this activator could improve insulin sensitivity and increase mitochondrial capacity in obese mice (Milne et al., 2007). As a result, these activators were proposed to have therapeutic effects in the management of type 2 diabetes. Interestingly, a very recent report tested the specificity of resveratrol and the SRT compounds against SIRT1

24

and found them to be functional only in Fluorescence-based assays where the peptide substrate of SIRT1 was covalently attached to a fluorophore. It was discovered that these compounds could directly bind to the fluorophore-peptide substrates and increase the affinity of the peptide for SIRT1. Neither the SRT compounds nor resveratrol were able to activate SIRT1 when native full length acetylated p53 peptide lacking the fluorophore was used as a substrate (Pacholec et al., 2010). These findings re-iterate the doubts concerning the ability of resveratrol to specifically activate SIRT1 since previous results from Borra et al., have suggested that SIRT1 activation by resveratrol with a coumarin labeled peptide occurs due to conformational change induced by resveratrol near the coumarin binding site in SIRT1. The conformational change creates a binding pocket for the coumarin group which enhances the binding of the coumarin labeled peptide to SIRT1 and resveratrol is unable to activate SIRT1 in the absence of the coumarin label (Borra et al., 2005).

SIRT1

Among the seven sirtuins, SIRT1 is the closest human homolog of yeast Sir2 gene. While Sir2 has been implicated in promoting longevity upon calorie restriction in lower organisms, the role of SIRT1 on this matter is quite unknown. But the potential for its involvement as a promoter of longevity makes it an attractive molecule to investigate. In the past decade, a number of SIRT1 functions have been discovered and its role in aging, diabetes and cancer has been studied extensively.

Structure of SIRT1. The *SIRT1* gene is located on chromosome 10q21 and encodes a 747 amino acid long protein. It consists of eight exons and the protein is localized in the nucleus. Characteristic of this class, SIRT1 requires NAD+ for its enzymatic activity which is subsequently metabolized to produce nicotinamide along with the deacetylated product and O acetyl-ADP ribose. The catalytic domain of SIRT1 that spans from 244 to 498 amino acids is

evolutionarily conserved and consists of two sub-domains. The large sub-domain resembles a Rossmann fold and the small sub-domain is composed of a helical module and a zinc-binding module (Figure 3). The interface between the two sub-domains creates a large grove that consists of the active site where NAD+ and the acetylated substrate bind (Huhtiniemi et al., 2006). While the mechanism of deacetylation of substrate has been resolved, it has been reported that SIRT1 shows no substrate specificity *in vitro*. The amino acids proximal to the acetylated lysine do not show any consensus sequences among the SIRT1 substrates (Blander et al., 2005). Unpublished work from our lab as well as reports from other labs have shown that the SIRT1 protein consists of about 13 sites that can be potentially phosphorylated (Sasaki et al., 2008). These phosphorylation sites are distributed all along the protein and the biological function of phosphorylated SIRT1 is yet to be determined. Thr-530 and ser-540 have been shown to be phosphorylated by cyclinB/cdk1 complex and CK2 has been found to phosphorylate ser-659 and ser-661 on SIRT1 (Sasaki et al., 2008; Zschoernig and Mahlknecht, 2009). There is also evidence that lys734 on SIRT1 can be sumoylated and this modification enhances the deacetylase activity of SIRT1 (Yang et al., 2007).

SIRT1 deacetylates histone H3 lys9, histone H4 lys16 and histone H1 lys26 and promotes the formation of facultative heterochromatin (Vaquero et al., 2004). It also acts as a non-histone protein deacetylase (Wojcik et al., 2009). The biological effects of SIRT1 mediated deacetylation and modulation of histone and non-histone substrates has been observed in aging, metabolism and cancer, as described below.

Small sub-domain
and
"Flexible Loop"

Large sub-domain
Rossmann Fold
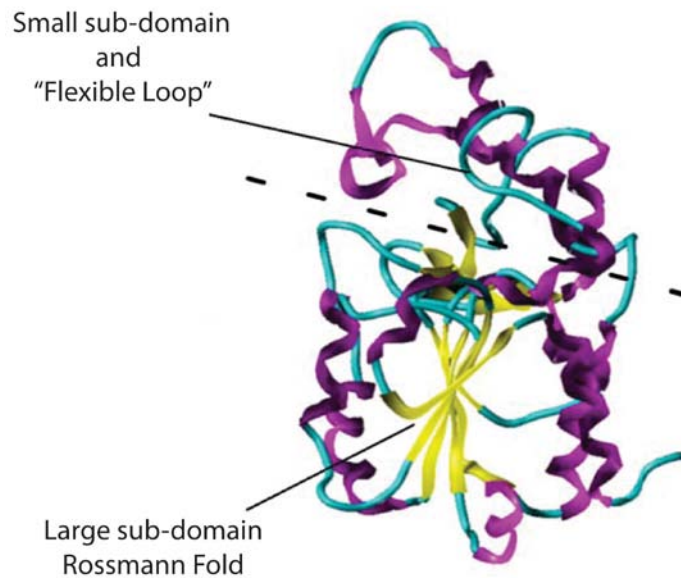
Fig 3: Structure of SIRT1 Catalytic Core. The interface between the two sub-domains is represented by the dash-line and that is where the active site of SIRT1 is located (Huhtiniemi et al., 2006).

SIRT1 in Aging and Metabolism. The role of SIRT1 in promoting longevity upon calorie restriction (CR) is well known in lower organisms (Gottlieb and Esposito, 1989; Lin et al., 2000; Tissenbaum and Guarente, 2001). Therefore, investigation of its role in human aging is currently under evaluation. With the discovery of the SIRT1 activator resveratrol, a polyphenol found in red wine, the function of this HDAC as an anti-aging molecule gained more interest and detailed research (Hall, 2003; Howitz et al., 2003). Restrictions in calorie intake have been linked to increase in life-span in a number of studies (Guarente, 2000; Roth et al., 1999; Weindruch et al., 1986). However, the effect of calorie restriction on human longevity is currently not fully understood. On the question of mechanism, several theories have been put forth to explain the relationship between calorie restriction and aging. Metabolic changes have been presented as an important player in this relation. According to recent research, CR activates SIRT1 and SIRT1, in turn, regulates proteins that are involved in metabolism and promotion of aging. Upon calorie restriction, the rate of respiration increases which leads to

27

an in increased rate of re-oxidation of NADH to generate NAD+. The increase in the ratio of NAD+/NADH, mainly due to a decrease in NADH levels, may be responsible for increased SIRT1 activity (Lin et al., 2002). It has also been shown that PNC1 (pyrazinamidase/ nicotinamidase-1) can extend life span upon calorie restriction via SIRT1 activation. PNC1 encodes an enzyme that deaminates nicotinamide and lowers its levels by converting it into nicotinic acid. Since nicotinamide is a known inhibitor of the enzymatic activity of SIRT1, decrease in its levels facilitates the activation of SIRT1 (Anderson et al., 2003). It has been reported that upon nutrient withdrawal, FOXO3a interacts with p53 and binds to *SIRT1* promoter thereby upregulating the transcription of SIRT1. Knockdown of FOXO3a or p53 inhibited the induction of SIRT1 upon starvation (Nemoto et al., 2004). Other reports have stated that SIRT1 protein levels increase upon calorie restriction but this increase is due to increased stability of the protein rather than increased transcription from the *SIRT1* gene (Kanfi et al., 2008a). Recently, it was reported that SIRT1 induction upon CR was tissue-specific (Chen et al., 2008). SIRT1 levels were higher in WAT (white adipose tissues) and muscles of calorie restricted mice as compared to *ad libitum* mice but lower in the liver of CR mice. WAT is the principal fat storage tissue and reduction in the storage of fat leads to increased lifespan in mammals. The ratio of NAD+/NADH was found to be higher in the WAT and muscles of CR mice but low in the liver which may be responsible for the variable extent of SIRT1 induction in different tissues. SIRT1 mediates the effect of calorie restriction by regulating a number of proteins involved in different metabolic pathways. SIRT1 has been shown to deacetylate and negatively regulate Peroxisome proliferator-activated receptor gamma 2 (PPARγ2), a key transcription factor that regulates the maintenance and differentiation of adipocytes and promotes adipogenesis (Picard et al., 2004; Tontonoz et al., 1994). SIRT1 acts as a co-repressor of PPARγ2 and has been found on the promoter of the PPARγ2 target gene, *aP2*. By deacetylating PPARγ2, SIRT1 prevents the accumulation of fat and promotes fat mobilization in WAT. Interestingly, in mice, SIRT1 was found to be recruited

to the PPARγ2 DNA-binding sites in WAT only after fasting indicating that the mobilization of fat was a result of CR mediated by SIRT1 (Picard et al., 2004). SIRT1 has been shown to control the gluconeogenic pathway in liver in response to calorie restriction by modulating a key transcriptional coactivator PGC-1α (Rodgers et al., 2005).SIRT1 interacts with PGC-1α and deacetylates it thereby activating the transcriptional function of PGC-1α in the liver as evidenced by the induction of gluconeogenic genes that express proteins such as PEPCK and glucose 6 phosphatase (G6Pase). While SIRT1 regulates gluconeogeneis in liver via deacetylation of PGC-1α, in the muscles and brown adipose tissues, it regulates mitochondrial biogenesis by deacetylating and activating PGC-1α. Enhanced mitochondrial activity results in resistance to diet-induced obesity (Lagouge et al., 2006). Upon low glucose availability (nutrient limitation), SIRT1 has been shown to induce mitochondrial fatty acid oxidation in skeletal muscles by deacetylating PGC-1α and activating its transcriptional target genes (Gerhart-Hines et al., 2007). Limiting levels of glucose induce SIRT1 by increasing NAD+ levels and SIRT1 maintains the bioenergetic state of the cell by switching from glucose to fatty acid metabolism. These reports suggest that SIRT1 regulates metabolism and homeostasis in response to CR in a tissue-specific manner. The transcription factor STAT3 has been shown to suppress PGC-1α in the liver thereby inhibiting gluconeogenesis. It has been reported that STAT3 phosphorylation and acetylation levels are low in the liver upon fasting and treatment with the SIRT1 inhibitor EX-527 brings back the levels of modified STAT3. In Sirt1 knockout MEFs, STAT3 acetylation and phosphorylation levels are constitutively high suggesting that STAT3 is regulated by SIRT1. STAT3 and SIRT1 form a complex and SIRT1 deacetylates STAT3 thereby suppressing the inhibitory effect of STAT3 on gluconeogenesis while activating PGC-1α, PEPCK and G6Pase (Nie et al., 2009). A mouse model overexpressing Sirt1 in pancreas was generated by a research group that suggested that SIRT1 expression in the β cells improved glucose tolerance and enhanced insulin secretion in response to glucose. These mice, called the BESTO mice (beta cell-

specific Sirt1-overexpressing), expressed reduced levels of UCP2, an uncoupling protein that

inhibits insulin, and increased ATP levels (Moynihan et al., 2005). Following studies

demonstrated that SIRT1 could repress the transcription of UCP2 by directly binding to the

UCP2 promoter (Bordone et al., 2006). However, 18-24 months old BESTO mice lost their

phenotype of glucose sensitivity and insulin secretion due to age-associated decline in

synthesis of NAD+ and loss of Sirt1 activity. Restoring NAD+ revived the glucose-tolerant

phenotype of the pancreatic β cells in these mice (Ramsey et al., 2008). Sirt1 overexpressing

transgenic mice also showed decreased levels of NF-κB, a pro-inflammatory transcription

factor that is deacetylated and inhibited by SIRT1. This inactivation of NF-kB prevents

cytokine-induced pancreatic β cell damage (Lee et al., 2009). It has also been shown that

induction of SIRT1, upon CR, imparts protection to cells from stress-induced death by

deacetylating Ku70 which in turn sequesters BAX in the cytoplasm and prevents apoptosis by

inhibiting its localization to the mitochondria (Cohen et al., 2004b). Therefore, SIRT1

promotes longer survival of cells.

Considering the beneficial effects of SIRT1 upregulation on metabolism, activators of SIRT1

are being extensively researched and developed. As mentioned earlier, resveratrol, a

polyphenolic metabolite found in grapes and grape products has been shown to extend life-

span in lower organisms. In mammals, it has been reported that resveratrol can activate

SIRT1 to enhance mitochondrial biogenesis by deacetylating and activating PGC-1α in

muscles and brown adipose tissues (Lagouge et al., 2006). It has also been reported that

resveratrol can change the physiology of older mice fed on a high-calorie diet to that of a

mouse fed on normal diet and increase the survival age of these mice by increasing insulin

sensitivity, decreasing IGF-1 levels and increasing PGC-1α activity thereby improving the

general health of the animals (Baur et al., 2006). Small molecule activators of SIRT1 are

being developed to specifically and significantly enhance the activity of SIRT1 and this

includes the recently published report on SRT1720 (Milne et al., 2007). As mentioned

previously, this small molecule activator is a 1000-fold more potent than resveratrol and when used in diet-induced obese mice, it is able to improve glucose homeostasis and insulin sensitivity in liver, fat and muscle. The activator is able to mimic the positive effects of calorie restriction on metabolism and homeostasis. However, as stated in the last section, recent reports have questioned the specificity of resveratrol and SRT compounds against SIRT1 and have shown their ability to interact with a number of different proteins (Borra et al., 2005; Pacholec et al., 2010). Hence, their effect on longevity and glucose homeostasis may not be specifically via activation of SIRT1.The importance of SIRT1 in affecting major metabolic pathways has led us to investigate its role, if any, in tumorigenesis. The majority of cancers show a direct correlation between incidence and age. Cancer has also been shown to be an outcome of deregulated metabolism. Therefore, investigation of the role of SIRT1 in cancer will undoubtedly provide insight into the molecular mechanisms of SIRT1 and may provide new clinical targets for the prevention and treatment of this disease. The following sections will follow the effect of SIRT1 on major transcriptional and translational signals and attempt to identify its function in cancer.

SIRT1 and Cancer

Histone deacetylases are better known as lysine deacetylases due to their ability to deacetylate histone as well as non-histone proteins. SIRT1 is known to have a number of histone and non-histone protein substrates and by modifying them via deacetylation, SIRT1 plays an essential role in the regulation of key signaling pathways (Figure 4). It is now well established that cancer is a result of deregulation of many of these pathways and the function of SIRT1 in manipulating proteins involved in these pathways is an active area of investigation.
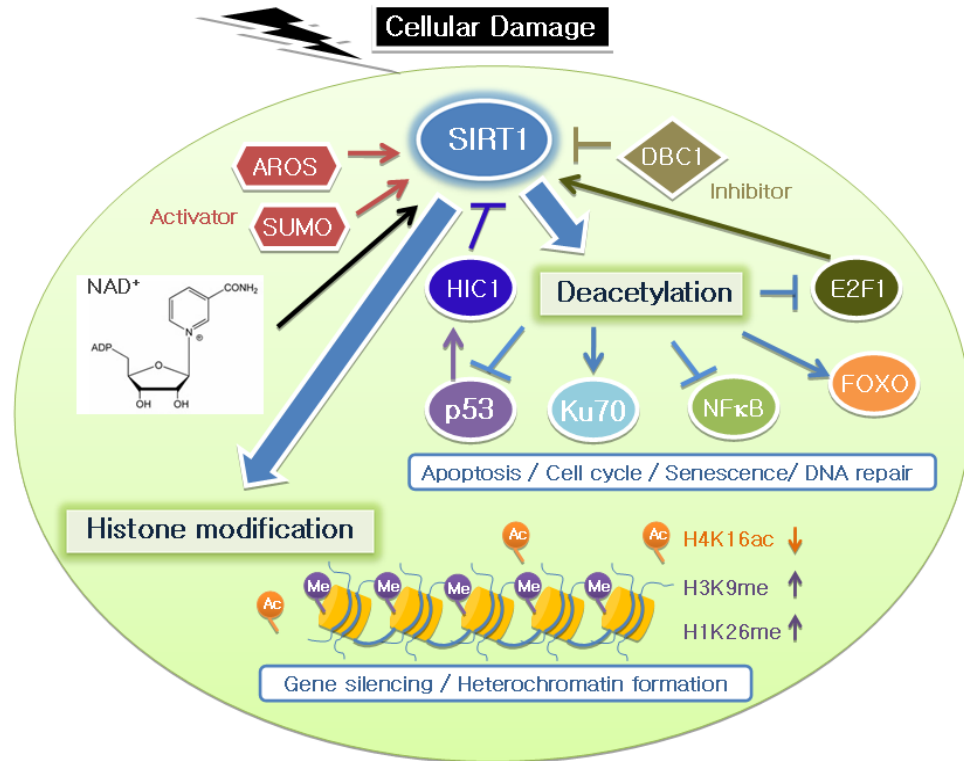
Fig 4: Functions of SIRT1. SIRT1 regulates various transcription factors (only few depicted in the diagram) involved in senescence, apoptosis and cell cycle progression. It also regulates gene silencing and heterochromatin formation by epigenetically regulating histones (Kim and Um, 2008).

SIRT1 Functions as a Histone Modifier and Gene Silencer. SIRT1 was discovered as a histone deacetylase and hence, its primary function as a histone modifier is well documented. SIRT1 has been shown to deacetylate lys9 of histone H3, lys16 of histone H4 and lys26 of histone H1 (Vaquero et al., 2004). Deacetylation of the above mentioned residues is a mark of condensed and genetically inactive DNA. SIRT1 also promotes methylation of histone H3 lys9 and loss of methylation on histone H3 lys79 which is interestingly, also a mark of repressed DNA (Vaquero et al., 2004). Such modifications in the promoters of genes results in silencing of transcription. Besides modifying the histones directly, SIRT1 has also been shown to deacetylate SUV39H1 and enhance its methyltransferase function to promote tri-

methylation of histone H3 lys9 thereby promoting condensation of chromatin (Vaquero et al., 2007). Recently, it was shown that SIRT1 formed a complex with SUV39H1 and Nucleomethylin (NML) upon low nutrient availability and this complex could bind to histone H3 lys9 in the rDNA locus resulting in deacetylation of histone H3 and dimethylation of lys9 ultimately repressing rRNA transcription. This silencing mechanism protects the cells from energy deprivation-dependent apoptosis (Murayama et al., 2008). p300/CBP are histone acetyltransferases (HAT) that are involved in acetylating histones and activating transcription. These HATs need to be acetylated themselves to be functional. SIRT1 was found to deacetylate these HATs and facilitate their SUMOylation thereby indirectly promoting hypoacetylation of histones (Bouras et al., 2005). With regards to cancer, deacetylation and methylation of histones in the promoters of tumor suppressor genes can lead to loss of expression of tumor suppressor proteins thereby making the cell susceptible to transformation. On the other hand, suppression of oncogene expression by gene silencing may support the role of SIRT1 as a tumor suppressor gene.

SIRT1 Deacetylates and Regulates Non-Histone Proteins. A number of transcription factors, repair proteins and signaling factors have been found to be substrates of SIRT1. With ongoing research, the list of non-histone substrates of SIRT1 is ever growing. It is interesting to note that SIRT1 regulates pro-apoptotic as well as anti-apoptotic proteins and this complicates the designation of *SIRT1* as an oncogene or tumor suppressor gene in cancer.

Pro-Apoptotic Proteins Deacetylated by SIRT1. P53 was among the first non-histone substrates of SIRT1 to be discovered. It was shown that SIRT1 could deacetylate p53, inhibit its transcriptional function and prevent cellular senescence and apoptosis induced upon stress and DNA damage (Luo et al., 2001; Smith, 2002; Vaziri et al., 2001). In this regard, SIRT1 was considered an oncogenic factor due to its ability to inhibit the pro-apoptotic

33

functions of p53. In subsequent years, research showed that while SIRT1 deacetylated p53, there was no biological outcome of this deacetylation in terms of expression of p53 target genes or cell survival in response to stress (Kamel et al., 2006; Solomon et al., 2006). Studies in mouse models also demonstrated that there was no difference in the phenotype of mice that were genetically null for Sirt1 or null for both Sirt1 and p53 and neither was there a difference in the response of the two genotypes to radiation induced apoptosis (Kamel et al., 2006). Another research group reported that embryonic fibroblast cells from Sirt1 null mice exhibited hyperacetylated p53 after DNA damage but this was not accompanied with increased p21 levels or DNA damage sensitivity (Cheng et al., 2003). These results question the oncogenic role of SIRT1 in cancer.

SIRT1 has been shown to deacetylate FOXO proteins. FOXO3a is an acetylated protein in a normal cell and this acetylation increases in response to stress such as exposure to hydrogen peroxide or growth factor deprivation. SIRT1 interacts with FOXO3a upon oxidative stress and deacetylates it. As a result, SIRT1 modulates the transcriptional function of FOXO3a. FOXO3a transcriptionally regulates a variety of genes that express proteins involved in DNA repair (GADD45), cell cycle arrest (p27) and apoptosis (Fas ligand, BIM). The effect of SIRT1 on FOXO3a can either promote cell cycle arrest and DNA repair or inhibit FOXO3a-dependent apoptosis in response to oxidative stress depending on the tissue-specific expression of the FOXO3a target genes (Brunet et al., 2004; Motta et al., 2004). Similarly, FOXO4, another member of the FOXO family is deacetylated by SIRT1. It was reported that upon oxidative stress, acetylation of FOXO4 by CBP inhibits its transcriptional function and deacetylation by SIRT1 rescues this inhibition (Motta et al., 2004; van der Horst et al., 2004). In prostate cancer cells, FOXO1, a member of the FOXO family of proteins, is deacetylated and inhibited by SIRT1 via interaction with FHL2 (Four and a half LIM 2). FOXO1 is known to interact with the insulin response sequences in *IGFBP1* gene promoter and transcriptionally

activate the gene but co-expression of FHL2 inhibits the activity of FOXO1. It was reported

that FHL2 enhances the interaction of SIRT1 and FOXO1 to facilitate deacetylation of FOXO1

and inhibit FOXO1-mediated apoptosis (Motta et al., 2004; Yang et al., 2005).

SIRT1 has also been shown to deacetylate and inhibit the transcriptional function of E2F1.

E2F1 has the unique property of behaving as a tumor suppressor gene as well as an

oncogene depending on the degree to which it is expressed and in the context of the cell

cycle before or after DNA damage (Crosby and Almasan, 2004; Yamasaki, 1999). Knocking

down SIRT1 has been shown to stimulate the transcriptional and apoptotic functions of E2F1

after treatment with DNA damaging drug etoposide. E2F1 on the other hand can

transcriptionally induce the expression of SIRT1 thereby forming a negative feed-back loop

(Wang et al., 2006).

A recent report suggests that SIRT1, acetyltransferase PCAF and E2F1 are all recruited to

the promoter of the E2F1 target p73. Upon increase in intracellular NAD+ levels, SIRT1

negatively regulates the acetylation and stability of PCAF as well as deacetylates E2F1 to

inhibit the transcription of p73 (Pediconi et al., 2009). It has also been shown that similar to

p53, SIRT1 can bind to p73 and deacetylate it thereby negatively regulating the pro-apoptotic

transcriptional function of p73 (Dai et al., 2007).

Retinoblastoma (*RB*) is a well known tumor suppressor gene that regulates cell cycle through

its interaction with the E2F family of transcription factors (Harbour and Dean, 2000; Zhu,

2005). The function of RB is modulated by posttranslational modifications such as

phosphorylation and acetylation. RB is hypophosphorylated and active during the quiescent

stage of the cell cycle and hyperphosphorylated and inactivated during active cycling of the

cell. Most cancers favor the hyperphosphorylated form of RB in order to maintain deregulated

cell proliferation. It has been shown that active RB is generally hypophosphorylated and

acetylated in the cell. SIRT1 can deacetylate RB and this deacetylation is associated with hyperphosphorylation of RB. Hence, SIRT1 promotes cell cycle progression by negatively regulating RB (Wong and Weber, 2007).

Anti-Apoptotic Proteins Deacetylated by SIRT1. NFκB is a transcription factor that regulates genes involved in cell cycle, adhesion, angiogenesis and apoptosis. Active NFκB is a heterodimer consisting of two subunits p50 and p65/RelA. It was shown that SIRT1 could interact with the p65/RelA subunit and deacetylate lys310 on this subunit. This deacetylation negatively affects the transactivation potential of NFκB. SIRT1 was also found on the promoter of NFκB target gene *BIRC3* which encodes the anti-apoptotic protein c-IAP2. The presence of SirT1 delayed the recruitment of NFκB to the *BIRC3* promoter thereby blocking NFκB-mediated transcription and facilitating apoptosis (Yeung et al., 2004).

Androgen receptor (AR) is a DNA binding transcription factor that governs cellular differentiation and proliferation in response to androgens in prostate cancer. AR activity is regulated by the hormone Dihydrotestosterone (DHT). DHT enhances co-activators such as p300 and SRC and reduces co-repressors such as NCoR, HDA and Smad association with AR. It was found that SIRT1 could associate with AR *in vitro* and *in vivo* and repress its transcriptional activity by deacetylating AR. Hence, SIRT1 can inhibit the tumorigenic phenotype of prostate cancer cells that are dependent on DHT and AR for proliferation (Fu et al., 2006).

β-catenin is a key component of the Wnt signaling pathway that regulates embryogenesis and is frequently deregulated in cancers. In the absence of the Wnt ligand, β-catenin is constitutively phosphorylated by GSK-3 and degraded. Upon Wnt binding to its receptor, GSK-3 is inhibited from binding to β-catenin which results in the stabilization and translocation

of β-catenin to the nucleus where it functions as a transcription factor to drive the expression of important genes such as *CCND1* (cyclin D1). β- catenin has been found to be constitutively activated in a majority of colon cancers. By overexpressing SIRT1 in the intestines of transgenic mice, it was shown that SIRT1 was capable of negatively regulating β-catenin. SIRT1 can deacetylate β-catenin and reduce its nuclear expression thereby inhibiting cell proliferation and oncogenic function of β-catenin (Firestein et al., 2008).

c-MYC belongs to a family of transcription factors that bind to the E-box sequences on the promoters of genes such as lactate dehydrogenase A (*LDHA*), *TERT*, etc., and activate transcription by recruiting histone acetyltransferases. c-MYC is often deregulated in cancers in response to oncogenic stimulation. It was shown that SIRT1 could deacetylate c-MYC protein at lys323 and decrease the stability of the protein. This in turn, decreased the expression of c-MYC target genes. In xenograft experiments, overexpression of SIRT1 inhibited tumorigenesis of myc3 cells that were injected in nude mice suggesting the tumor suppressive role of SIRT1. Interestingly, c-MYC was found to transcriptionally activate SIRT1 by binding to the E-box sequences in the *SIRT1* promoter thereby forming a negative feed-back loop with SIRT1 (Yuan et al., 2009).

As mentioned previously, SIRT1 can deacetylate and inhibit the transcriptional function of E2F1 (Wang et al., 2006). Under certain conditions, overexpression of E2F1 promotes cell proliferation and tumorigenesis and by deacetylating E2F1, SirT1 can rescue the oncogenic phenotype of E2F1.

SIRT1 in DNA Damage Repair. It has been hypothesized that SIRT1 can affect DNA damage repair by modifying the chromatin structure at the site of damage. An increase in nicotinamide at the site of damage due to activation of PARP (NAD-dependent enzyme) can

result in the temporary inactivation of SIRT1 at the DNA damage site thereby allowing for the deacetylated chromatin to de-condense and grant access to repair enzymes (Kruszewski and Szumiel, 2005).

Ku70 is a well known DNA damage repair factor that is involved in repairing double strand breaks in the DNA caused by ionizing radiations, physiological oxidation reactions, chemotherapeutic drugs, etc. It has been reported that upon acute stress, Ku70 is acetylated at lys539 and lys542 by CBP/PCAF acetyltransferases and this acetylation is responsible for disrupting the association of Ku70 with the proapoptotic factor BAX. As a result BAX localizes to the mitochondria to bring about apoptosis (Cohen et al., 2004a). It was shown that upon calorie restriction, SIRT1 was induced and it deacetylated Ku70. Upon deacetylation, Ku70 associated with BAX and sequestered it in the cytoplasm thereby preventing its localization to the mitochondria and preventing apoptosis. Hence, by regulating the DNA repair protein Ku70, SIRT1 was shown to promote cell survival (Cohen et al., 2004b).

The MRN (MRE11-RAD50-NBS1) is a DNA repair complex that localizes to DNA strand breaks and activates the DNA damage checkpoints for repair. NBS1 is the regulatory subunit of the MRN complex and it is phosphorylated by ATM kinase upon DNA damage. This phosphorylation is important for sensing of damage by the MRN complex and efficient repair response. p300 and PCAF acetyltransferases were reported to acetylate NBS1 and acetylation of NBS1 inhibited its phosphorylation. Of all the histone deacetylases, only SIRT1 was found to deacetylate NBS1. Hypoacetylated NBS1 could be phosphorylated at ser-343 in response to ionizing radiation to mediate DNA repair (Yuan and Seto, 2007). Hence, SIRT1 was found to promote DNA damage repair by positively regulating NBS1.

SIRT1 and Cellular Senescence. Mouse embryonic fibroblasts (MEFs) harvested form mice that were genetically null for Sirt1 exhibited different properties as compared to wild type fibroblasts. It was found that after a few passages, wild type MEFs underwent growth arrest while Sirt1 null MEFs continued to proliferate. Adding back Sirt1 caused the MEFs to behave similar to the wild type MEFs. Interestingly, adding back deacetylase mutant of Sirt1 did not revert the null phenotype to wild type suggesting that Sirt1 deacetylase activity was important for reversal of the enhanced proliferation of the null phenotype. It was also shown that p19ARF levels accumulated in MEFs over several passages but the accumulation was significantly less in the Sirt1 null MEFs. It was also shown that knock down of SIRT1 in human primary fibroblasts could extend their replicative lifespan. However, SIRT1 has not been shown to deacetylate p19ARF or the histones on its promoter. The targets of SIRT1 that influence replicative senescence have not been identified (Chua et al., 2005).

SIRT1 in Autophagy. Autophagy is a regulated mechanism used to degrade organelles and proteins in order to maintain homeostasis and normal development of the cell. It is known to be induced upon nutrient withdrawal to shut down non-essential processes and conserve energy for the essential pathways required for cellular survival. Under extreme stress, however, autophagy functions as another mode of cell death besides apoptosis, necrosis and senescence. *ATG* genes play an important role in mediating autophagy via formation of autophagosomes. Several *ATG* genes are known and it has been shown that SIRT1 can form a molecular complex with ATG 5, 7 and 8 and deacetylate them (Lee et al., 2008; Salminen and Kaarniranta, 2009). Sirt1 null mice exhibited hyperacetylation of the ATG proteins and these mice were unable to induce autophagy in the event of starvation. Accumulation of defective organelles and misfolded proteins were observed in the tissues of the Sirt1 null mice. Transient overexpression of Sirt1 induced autophagy in cells despite the presence of nutrients while the deacetylase mutant of Sirt1 was unable to do so indicating the importance

of the deacetylase activity of Sirt1. All in all, the above results demonstrated that upon calorie restriction, Sirt1 was induced which in turn upregulated the basal levels of autophagy in the cell to conserve energy and maintain survival of the cell upon stress (Lee et al., 2008; Salminen and Kaarniranta, 2009).

Mouse Models of SIRT1

In 2003, a Sirt1 null mouse model was generated by Cheng et al., to study the role of SIRT1 in normal growth and development. By gene-targeting, mice were generated that expressed either a mutant Sirt1 protein that lacked part of the catalytic domain or did not express Sirt1 at all. It was observed that both mice exhibited the same phenotype. Only 10% of the Sirt1 null mice were obtained due to a high rate of postnatal lethality for this genotype. The surviving Sirt1 null mice were smaller than the control littermates with very low sperm counts, cardiac defects and eye abnormalities. Of note was the observation that upon DNA damage by UV-radiation or adriamycin, p53 was hyperacetylated in the Sirt1 null MEFs as compared to the control MEFs but the hyperacetylation was not accompanied by an increase in the total levels of p53 or phosphorylated levels of p53 nor was there any difference in the levels of p21 protein as compared to control MEFs. There was no difference in sensitivity to ionizing radiation between the Sirt1 null and wild type MEFs. However, thymocytes from Sirt1 null mice were found to be more sensitive to ionizing radiation in a p53-dependent manner as compared to those from the wild type control mice (Cheng et al., 2003).

Another Sirt1 transgenic mouse was developed recently by Wang et al., to study the function of SIRT1 in tumorigenesis. Sirt1 null mice were generated by deleting exons 5 and 6 from the *Sirt1* gene resulting in loss of Sirt1 protein expression. The postnatal mortality of the Sirt1 null mice was higher than that observed in the mouse model generated by Cheng et al., most likely due to the difference in the mouse strain. Chromosomal abnormalities and incomplete chromosomal condensation was observed in the Sirt1-deficient cells as was a lack in

heterochromatin formation. The cells exhibited abnormalities in cell cycle check-points at low

doses of γ radiation and defects in DNA damage repair. Sirt1 deficient cells also exhibited

fewer γH2AX foci formation in response to ionizing radiation indicating impaired DNA damage

repair. Transfection of *Sirt1* in these Sirt1 null cells resulted in the formation of almost equal

numbers of γH2AX foci as wild type cells. *Sirt1-/-;p53-/-* mice were generated and it was

found that absence of p53 could not rescue the phenotype associated with Sirt1 deficiency

suggesting that Sirt1 null phenotypes are not caused by p53 activation. It was also found that

Sirt1+/-;p53+/- mice developed spontaneous tumors and the tumor incidences were much

higher than those observed in *Sirt1+/-* or *p53+/-* mice. The tumors from the double

heterozygous mice had severe chromosomal abnormalities and while most of the tumors

exhibited LOH for p53, majority of them retained one allele of *Sirt1* suggesting that *SIRT1*

may function as a haploid tumor suppressor gene (Wang et al., 2008a).

Regulation of SIRT1

SIRT1 is regulated at transcriptional, posttranscriptional and posttranslational levels. While

the functions of SIRT1 have been extensively studied and described, there is a lack of

comprehensive information on its regulation. A few proteins have been reported to modulate

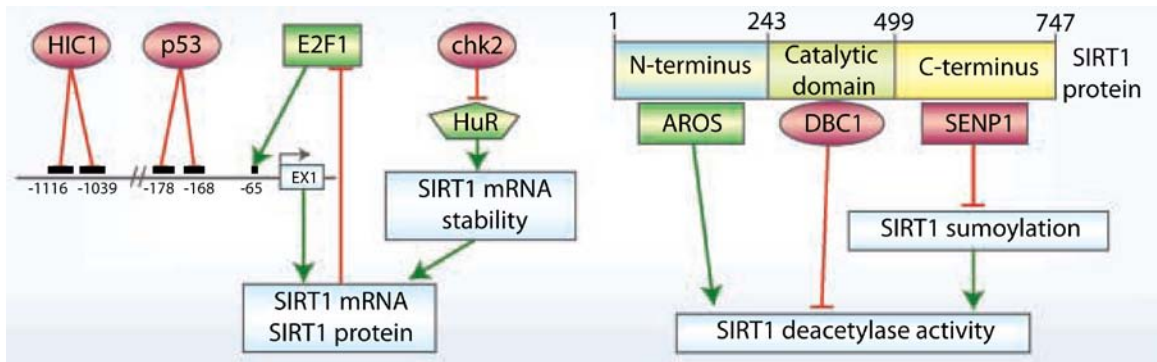the expression and function of SIRT1 as stated below.

Fig 5: Regulation of SIRT1. *SIRT1* gene expression is modulated at both transcriptional and posttranscriptional levels, and its deacetylase activity is modulated through protein-protein interaction and sumoylation at its three protein domains (Liu et al., 2009).

Transcriptional Regulation of SIRT1. The earliest reports on SIRT1 regulation involved FOXO3a and p53 (Nemoto et al., 2004). It was shown that upon nutrient deprivation, *SIRT1* mRNA as well as protein levels were induced up to four fold and this induction was associated with nuclear translocation of the transcription factor FOXO3a. Knock down of FOXO3a abrogated the induction of SIRT1 expression levels whereas constitutive activation of FOXO3a resulted in activation of *SIRT1* promoter despite the presence of nutrients. It was further shown that the *SIRT1* promoter contained two p53 binding sites and mutation of either one of them reduced the stimulatory effect of nutrient deprivation on SIRT1. FOXO3a and p53 were found to interact with each other upon nutrient withdrawal and FOXO3a stimulated *SIRT1* mRNA expression via p53 binding sites on the *SIRT1* promoter (Nemoto et al., 2004).

E2F1 has been shown to induce *SIRT1* gene transcription. As mentioned earlier, E2F1 forms a negative feed-back loop with SIRT1. SIRT1 deacetylates and inhibits E2F1 transcriptional function while E2F1 can bind to *SIRT1* promoter at around 65 bp upstream of the ATG sequence and induce *SIRT1* gene expression. The E2F1/RB pathway is frequently deregulated during oncogenic transformation and increased activation of E2F1 leads to

increase in SIRT1 expression in cancer. It was also shown that DNA damage induced by drugs such as etoposide resulted in stabilization of E2F1 and was associated with enhanced expression of *SIRT1* mRNA and protein levels. Hence, high SIRT1 levels may be an outcome of transformation rather than its cause (Wang et al., 2006).

HIC1 or Hypermethylated in Cancer 1 is a transcriptional repressor that is frequently silenced in cancer by epigenetic modifications. It has been shown to interact with p53 and function as a tumor suppressor gene that can induce cell death upon DNA damage. HIC1 has a POZ domain that allows for protein-protein interaction and it is through this domain that it can interact with SIRT1. In HIC1 null cells, SIRT1 levels were elevated. It has been shown that HIC1 can interact with SIRT1 protein and HIC1-SIRT1 complex can be recruited to the *SIRT1* promoter to repress the transcription of *SIRT1* (Chen et al., 2005). As a result of transcriptional repression of *SIRT1*, p53 is acetylated and stabilized to induce cell death upon DNA damage. With age, the HIC1 promoter undergoes hypermethylation and epigenetic silencing of gene expression resulting in upregulation of SIRT1 expression, deacetylation of p53 and resistance to DNA damage-induced cell death. Hence, SIRT1 promotes cell survival as well as the risk of transformation. HIC1 has also been shown to interact with CtBP, a transcriptional corepressor, to downregulate *SIRT1* gene expression. Upon stress that increases the NAD+/NADH ratio, CtBP undergoes certain posttranslational modifications and its interaction with the proline-rich domain of HIC1 decreases. Due to this decreased association, HIC1 is unable to repress *SIRT1* gene transcription effectively and SIRT1 expression is induced (Zhang et al., 2007).

The oncogene c-MYC has also been found to transcriptionally regulate SIRT1 (not shown in Figure 5). c-MYC forms a negative feedback loop with SIRT1 wherein SIRT1 deacetylates c-MYC protein and decreases its stability while c-MYC binds to the *SIRT1* promoter and

induces its transcription (Yuan et al., 2009). The *SIRT1* promoter was found to contain three potential c-MYC binding regions (E-Box 1, 2 and 3). By ChIP analysis, it was found that c-MYC localized to the E-box (E1) on the *SIRT1* promoter and enhanced *SIRT1* transcription. Upon oncogenic transformation, deregulation and overexpression of c-MYC is often observed and this can lead to increase in SIRT1 levels in cancers. Increase in SIRT1 levels can, in turn, deacetylate and destabilize c-MYC thereby preventing the transcription of c-MYC target genes such as *TERT*. However, the tumor suppressive qualities of SIRT1 may be relevant only if the tumor has not become addicted to SIRT1 due to its anti-apoptotic functions (as described in the earlier sections).

Breast-Cancer-Associated-Gene 1 or *BRCA1* is a well known tumor suppressor gene which is frequently mutated in familial breast cancers. This gene plays an essential role in several important biological pathways involving DNA damage repair, cell cycle progression, apoptosis, etc. Recently, this gene has been shown to regulate the expression of SIRT1. It was noted that tumors exhibiting low levels of functional BRCA1 also had low levels of SIRT1 but higher levels of Survivin, an anti-apoptotic molecule. BRCA1 was found to transcriptionally upregulate SIRT1. Further experiments showed that overexpression of SIRT1 in BRCA1 mutant cancers could inhibit tumor formation by inhibiting cell proliferation. Interestingly, this inhibition of cell proliferation by SIRT1 was only seen in BRCA1 mutant cell lines and not BRCA1 wild type cells lines. It was also shown that SIRT1 could bind to the promoter of Survivin and promote methylation of histone H3 lys9 thereby inhibiting the transcription of Survivin. Therefore, BRCA1 was found to downregulate Survivin via regulation of SIRT1 (Wang et al., 2008b).

Posttranscriptional Regulation of SIRT1. The RNA binding protein HuR is the only protein that has been shown to regulate SIRT1 at the posttranscriptional level. It binds to the 3'

untranslated region of the *SIRT1* mRNA and stabilizes the transcript which leads to increased

SIRT1 protein expression (Abdelmohsen et al., 2007). In human diploid fibroblast cells, HuR

levels decrease upon senescence and this is associated with lower SIRT1 levels. Further, it

has been shown that upon oxidative stress, HuR is phosphorylated by the check-point kinase,

CHK2, and this modification causes HuR to dissociate from SIRT1. As a result, *SIRT1* mRNA

degrades and SIRT1 protein expression is downregulated thereby sensitizing the cells to

oxidative stress. Hence, HuR downregulates SIRT1 expression by affecting the stability of

SIRT1 mRNA after oxidative damage.

Posttranslational Regulation of SIRT1. The Active Regulator of SIRT1, *AROS*, was

identified from a yeast two-hybrid screen using SIRT1 as bait. AROS was found to bind to the

N-terminal sequence of SIRT1 and enhance SIRT1-mediated deacetylation of p53. By

promoting p53 deacetylation, AROS downregulated p53 transcriptional function as concluded

from p53-dependent BAX-luciferase and p21-luciferase reporter assays. Furthermore, SIRT1

inhibitors such as nicotinamide abrogated AROS and SIRT1–induced p53 inactivation.

Depletion of AROS resulted in hyperacetylated p53 and increase in p21 levels. It was also

found that cells stably expressing AROS were resistant to DNA damage caused by drugs

such as etoposide. This was due to the promotion of SIRT1 deacetylase activity which leads

to downregulation of p53 transcriptional function and loss of expression of p53 target genes

that promote cell death in response to stress and DNA damage (Kim et al., 2007; Verdin,

2007).

Deleted in Breast Cancer 1 or *DBC1* is a gene that is frequently lost in breast cancer due to

its location on chromosome 8p21 which shows LOH in breast cancer. Its function in the cell is

relatively unknown. Recently, it was shown that DBC1 could bind to SIRT1 and inhibit its

deacetylase activity (Kim et al., 2008; Zhao et al., 2008). The leucine zipper motif (LZ) of

DBC1 was found to bind to the N-terminal domain of SIRT1 and this association negatively regulated the deacetylase activity of SIRT1. Also, DBC1 was able to rescue SIRT1-mediated deacetylation of p53 by abrogating the SIRT1-p53 interaction (Zhao et al., 2008). It has also been shown that DBC1 can abrogate the SIRT1-SUV39H1 complex thereby affecting histone methylation and heterochromatin formation (Li et al., 2009).

Recent research has shown that the SIRT1 protein is sumoylated. SUMO (small ubiquitin related modifiers) molecules can tether to lysine residues of proteins similar to ubiquitin molecules but instead of targeting the sumoylated molecule for degradation, this modification plays a role in genomic stability and transcriptional silencing (Muller et al., 2004; Verger et al., 2003). Sumoylation is reversible and SENP1 functions as a sumo-specific protease to reverse the modification (Bailey and O'Hare, 2004). SIRT1 was found to be sumoylated at lys734 and this increased its deacetylase activity. SENP1 was found to desumoylate SIRT1 and downregulate its deacetylase activity. Stress-inducing agents such as UV radiation or hydrogen peroxide, increased the interaction of SENP1 with SIRT1 thereby promoting the desumoylation and inactivation of the anti-apoptotic function of SIRT1. Therefore, SENP1 was shown to regulate cell death in response to stress by activating p53 and its target genes via inhibition of SIRT1 activity (Yang et al., 2007).

Thirteen phosphorylation sites have been identified on the SIRT1 protein by Mass spectrometry (Sasaki et al., 2008). It has been shown that cyclin B/cdk1 can form a complex with SIRT1 and phosphorylate thr-530 and ser-540. Phosphorylation at these sites can increase SIRT1 activity in mitotic cells. Dephosphorylation of SIRT1 inhibits its deacetylase activity and fails to rescue the proliferation defects in Sirt1 null MEFs. However, the molecular targets that are affected by SIRT1 phosphorylation are still to be determined. Another report suggests that SIRT1 can be phosphorylated by CK2 at serine residues 154, 649, 651, 683

upon DNA damage. Phosphorylation of SIRT1 by CK2 increases its deacetylase activity by increasing its substrate binding affinity (Kang et al., 2009).

SIRT1 Expression in Cancer: Reports and Controversies

The earliest reports of SIRT1 as a non-histone deacetylase involved p53. It was shown that deacetylation of p53 by SIRT1 inhibited its transcriptional function and hence, *SIRT1* was considered an oncogene (Luo et al., 2001; Smith, 2002; Vaziri et al., 2001). The role of SIRT1 since then has been studied in detail and a number of SIRT1 functions have been brought to light. While some reports have deemed *SIRT1* as an oncogene based on its function and expression pattern in tumors (Huffman et al., 2007; Stunkel et al., 2007), other reports have provided evidence for a tumor suppressive function of SIRT1 (Firestein et al., 2008; Wang et al., 2008a). The importance of p53 acetylation in its stabilization and transcriptional function has been questioned. While some researchers have shown that acetylation of p53 mediated by p300/CBP in response to DNA damage enhances sequence-specific DNA binding (Lill et al., 1997; Olsson et al., 2007; Sakaguchi et al., 1998), others have shown that enhancement of p53 function is independent of its acetylation status (Espinosa and Emerson, 2001). In light of the above conclusions, the significance of p53 acetylation is controversial and so is the functional effect of SIRT1-mediated p53 deacetylation. Besides p53, SIRT1 has been shown to regulate proteins that participate in various pro-apoptotic and anti-apoptotic pathways. To date the controversies regarding the exact role of SIRT1 in cancer have not been resolved and further research will be required to reach a conclusion.

Stunkel et al., compared the levels of SIRT1 in cancer cell lines with normal cells and found that SIRT1 was overexpressed in almost all the cancer cell lines that were tested. When stained with anti-SIRT1 antibody, HeLa and SW620 cell lines exhibited cytoplasmic localization of SIRT1. Staining of a colon tumor microarray (TMA) also revealed cytoplasmic localization of SIRT1 in the tumor as well as normal colon tissues (Stunkel et al., 2007). This

result was unexpected since the nuclear localization of SIRT1 is a well established fact and further investigation of this expression pattern will reveal its significance. Another research group identified two nuclear import and two export sequences on SIRT1 protein and found that SIRT1 could shuttle between the nucleus and cytoplasm in C2C12 myoblast cells (Tanno et al., 2007). SIRT1 was nuclear in undifferentiated C2C12 and localized to the cytoplasm upon differentiation. Nuclear SIRT1 was found to protect the cells against oxidative damage-induced cell death but the function of cytoplasmic SIRT1 is yet to be determined. It was hypothesized that the cytoplasmic localization of SIRT1 is a mode of regulation such that removal of SIRT1 from the nucleus allows for acetylation and activation of certain nuclear substrates of SIRT1. To study the role of SIRT1 in cancer, Huffman, et al., developed a prostate cancer mouse model called TRAMP (Transgenic adenocarcinoma of mouse prostate) (Huffman et al., 2007). Sirt1 levels were found to be elevated in prostate adenocarcinomas in these mice. Simultaneously, HIC1 levels were downregulated leading the authors to suggest that lower levels of HIC1 expression were responsible for the observed increase in SIRT1. Levels of acetylated lys9 on histone H3 were also reduced in these cancers. Upon staining prostate tumor biopsies, it was found that SIRT1 expression was high in the cancer cells as compared to the normal surrounding cells. Based on the association of elevated SIRT1 levels with advanced prostate cancer, the authors summarized that SIRT1 functions as an oncogene and may serve as a potential target for prostate cancer therapy. However, this model does not provide conclusive evidence for SIRT1 as a cause of prostate cancer. It merely shows an association between high SIRT1 expression and prostate cancer. Recently, a research group generated a Sirt1 null mouse model to determine the role of SIRT1 in tumorigenesis (Wang et al., 2008a). They discovered that cells obtained from *Sirt1-/-* embryos exhibited incomplete chromosome condensation and chromosome instability. These cells also demonstrated cell cycle abnormalities and impaired DNA damage repair as compared to *Sirt1+/+* cells. Furthermore, it was discovered that *Sirt1+/-;p53+/-* mice were

more prone to development of spontaneous tumors as compared to *Sirt1+/-* or *p53+/-* mice. These observations led the authors to suggest that SIRT1 may function as a tumor suppressor. They then analyzed a set of clinical tissues to study the levels of SIRT1 expression in tumors and found that SIRT1 levels were lower than normal in glioblastoma, bladder carcinoma, prostate carcinoma and certain ovarian cancers. This expression pattern in the different tumors along with observations from the Sirt1 null mice led the authors to suggest that SIRT1 has a tumor suppressive function.

To study the effect of SIRT1 on tumor formation and growth in colon, the Apc^{Min} colon cancer mouse model was bred to a Sirt1 transgenic mouse to obtain progenies that overexpress Sirt1. Polyp formation in Sirt1 overexpressing mice was compared to control Apc^{Min} mice. As observed by Firestein, et al., Sirt1 overexpression was associated with fewer adenomas in the colon of Apc^{Min} mice suggesting a tumor suppressive role for Sirt1.It was shown that Sirt1 inhibited colon tumorigenesis by deacetylating and negatively regulating the oncogene, β-catenin. Indeed, in a colon TMA, the nuclear localization of SIRT1 was associated with a cytoplasmic localization of β-catenin suggesting that SIRT1 inhibits β-catenin by nuclear exclusion besides deacetylating and inhibiting its transcriptional function (Firestein et al., 2008).

Most recently, the McBurney lab generated a transgenic mouse model for Sirt1 null genotype. Most of these transgenic mice grew to adulthood probably due to their outbred background and were used to test whether SIRT1 inhibits or promotes the development of cancer (Boily et al., 2009). To study the effect of SIRT1 on skin carcinogenesis, DMBA was applied to the skin to initiate cancer and TPA to promote the cancer in wild type and Sirt1 null mice. No difference in tumor formation was observed between the two genotypes but interestingly, treatment with resveratrol could reduce the tumors in both groups although to a lower extent in Sirt1 null genotypes suggesting a SIRT1-dependent as well as independent anti-tumorigenic role of resveratrol. Upon crossing Sirt1 transgenic mice with Apc^{Min} mice to study

the effect of Sirt1 in colon cancer, it was found that the number of polyps that developed in the *Apc^Min^;Sirt1+/+* and *Apc^Min^;Sirt1-/-* mice were almost the same but the *Apc^Min^;Sirt1-/-* mice had smaller sized polyps. All in all, work by this research group concluded that SIRT1, at its endogenous expression level, does not modulate susceptibility to tumor formation but is essential for resveratrol-mediated chemoprotection and that the *SIRT1* gene expression may have to be induced to a certain level for it to function as a tumor suppressor.

The above observations concerning the role of SIRT1 in cancer are conflicting at best. Ongoing research is still attempting to answer whether *SIRT1* is a tumor promoter or tumor suppressor gene. Our investigation on this subject has provided more clues towards determining the role of SIRT1 in tumorigenesis. Our studies mainly focus on the role of SirT1 in colon cancer. Colon cancer is the third most common cancer in men and women in the United States. It is the second leading cancer killer but as many as 60% deaths from this cancer can be prevented by regular screening tests of individuals 50 years of age and older (Centers for disease control and prevention, 2009). Adenomatous polyps in the colon represent the benign form of this cancer. It is followed by adenomas with high-grade dysplasia and then invasive cancer. Stages I and II of the invasive cancer are confined within the walls of the colon. Further metastasis to the lymphnodes qualifies as stage III of colon cancer and metastasis to distant sites is considered stage IV (Markowitz and Bertagnolli, 2009). Stages I to III are curable by surgery and chemotherapy but stage IV disease remains incurable (Markowitz and Bertagnolli, 2009). 5-Fluorouracil (5-FU) is one of most commonly used drugs for the treatment of colon cancer. It is a nucleoside analog that blocks thymidylate synthase enzyme thereby inhibiting thymine nucleotide synthesis and preventing DNA replication. The vitamin Leucovorin is often administered with 5-FU to improve its efficacy as a therapeutic drug (Segal and Saltz, 2009). Camptothecin or CPT, a topoisomerase I inhibitor, was also found to be an effective agent against colon cancer although, a derivative of CPT called Irinotecan is more commonly used (Conti et al., 1996; Hsiang et al., 1985). Some

therapeutic regimens also involve a combination of Irinotecan and 5-FU as a more effective method of treatment (Douillard et al., 2000; Saltz et al., 2000).While early detection and newer drugs are helpful in reducing the number of deaths, morbidity and mortality due to colon cancer is still distressingly high and there is a constant need for identification of new markers and development of drugs against new targets to combat this disease.

Our experiments with SIRT1 have shown an interesting behavior of this protein in colon cancer. We have observed that SIRT1 inhibits proliferation of colon cancer cells and inhibiting SIRT1 by shRNA promotes tumor growth in xenograft experiments. Interestingly, inhibition of SIRT1 also sensitizes the cells to chemotherapy. Upon examination of a colon tumor microarray, SIRT1 was found to be highly expressed in normal colon mucosa and benign adenomas but its expression was low in a subset of malignant adenocarcinomas. Our results therefore, suggest a context-dependent tumor suppressive function of SIRT1 in colon cancer where SIRT1 inhibits tumor initiation and growth but augments cell death upon treatment with DNA-damaging drugs. Further studies in this direction will allow us to understand the importance of SIRT1 activators and inhibitors in the treatment of colon cancer.

Chapter Two

Materials and Methods

Cell Lines

HCT116 (kindly provided by Dr. Bert Vogelstein), U2OS, A549 and 293T cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). E2F1 null and wild type MEF cells lines were provided by Dr. Jack Pledger and Sirt1 null and wild type cell lines were generated by crossing Sirt1 heterozygous mice and generating MEFs from 13dpc embryos (protocol described in later sections). SIRT1 knock down in HCT116 cells was accomplished by using RNAi technology. A double-stranded oligonucleotide (5'-GATCCCG<u>TTGGATGATATGACA CTG</u>TTCAAGAGA<u>CAGTGTCATATCATCCAAC</u>TTTTTTTGGAAA , SIRT1 target sequence underlined) was cloned into the pSuperiorRetroPuro vector (OligoEngine). The plasmid was packaged into retrovirus by transfecting the amphotropic packaging cell line LA (a kind gift from Dr. Peiqing Sun, the Scripps Institute). Retrovirus containing supernatant from the transfected LA cells was used to infect HCT116 cells. The infected cells were selected in 0.5-1µg/ml puromycin for 10 to 14 days and the drug-resistant colonies were pooled for analysis. Virus expressing scrambled shRNA (5'-GATCCC <u>GCCGTCGTCGATAAGCAATAT</u>TTGATATCCG<u>ATATTGCTTATCGACGACGGC</u>TTTTTTA) was also generated and used to infect HCT116 cells that served as control. Another set of SIRT1 knock down HCT116 cell lines were generated using *SIRT1* shRNA in pSM2 vector (Openbiosystems shRNA library from Molecular Biology Core, H. Lee Moffitt Cancer Center). LA cells were used to generate virus expressing *SIRT1* siRNA and scrambled siRNA and these virus were used to infect HCT116 cells. Puromycin resistant colonies were pooled at

the end of two weeks and analyzed for SIRT1 knock down. SIRT1 knock down in A549 cells was obtained by the same protocol as described for HCT116 cells.

For the generation of tetracycline-inducible SIRT1 cell lines, a lentiviral system was used. Lentivirus vector expressing SIRT1 was generated using the ViraPower™ T-REx™ system following instructions from the manufacturer (Invitrogen). HCT116 were first infected with the T-REX regulator lentivirus and selected with 4 µg/ml blasticidin. This was followed by infection with the SIRT1 lentivirus and selection with 300µg/ml Zeocin to obtain a pool of colonies. SIRT1 overexpression was obtained upon induction with 1µg/ml tetracycline.

Plasmids and Reagents

Human SIRT1 plasmid was kindly provided by Dr. Wei Gu. The SIRT1 nuclear import mutant was generated by site-directed mutagenesis (K35E) in the putative NLS sequence RKRPRR. The deacetylase–dead mutant was generated by site-directed mutagenesis (H363A) in the catalytic domain of SIRT1. HA-tagged E2F1 plasmid was provided by Dr. Douglas Cress and HA-tagged CBP was provided by Dr. Hua Lu. SIRT1 monoclonal antibody 10E4, used for immunohistochemical staining was generated in the lab against the C-terminal domain of SIRT1. SIRT1 polyclonal antibody was purchased from Upstate Biotechnology. Monoclonal anti-E2F1 antibody (KH-95) was purchased from Santa Cruz Biotechnologies and antibodies against pRB and RB were purchased from Cell Signaling and Santa Cruz Biotechnology respectively. Polyclonal Antibody against E2F1 AcK117 was raised against acetylated E2F1 peptide HPG(AcK117)GVKSPG and affinity-purified. Anti-HA antibody was purchased from Covance. The SIRT1 inhibitor EX-527 was purchased from Tocris Bioscience. 5-FU (5 Fluorouracil) and CPT (Camptothecin) were purchased from Sigma and used at the indicated concentrations. Methyl-^{3}H thymidine was purchased from Amersham Biosciences for proliferation assays.

Transfections

Calcium Phosphate Transfections. 293T cells were used to perform calcium phosphate transfections because of their high transfection efficiency. To study the effect of SIRT1 on E2F1 acetylation, 293T cells were grown to almost 90% confluency in 10cm tissue culture dishes. A mixture of E2F1, SIRT1 and CBP (CREB binding protein) plasmids were made to 40µg and combined with 400µl water and 125mM calcium chloride. This mixture was added drop-wise to 500µl of HEPES (0.28 M NaCl, 0.05 M HEPES, 1.5mM CaCl2) while bubbling. After bubbling, the entire mixture (almost 1ml) was added to the plate of 293T and incubated for 16h-18h at 37°C. After 18h, the transfected 293T were re-fed with fresh medium and plates were incubated for another 24h before harvesting.

Lipofectamine Transfections. For transfection of cells in 6-well plates (colony formation assay) or 24-well plates (luciferase assay), Lipofectamine 2000 was used (Invitrogen). Cells were grown to 60% confluency in DMEM with 10% FBS but no antibiotics. For each transfection, required plasmids were mixed up to 1µg (including carrier DNA and GFP) and combined with 50µl OPTIMEM (reduced serum medium). To this plasmid mix, 1µl of Lipofectamine 2000 combined with 50µl of OPTIMEM was added and the entire mixture (almost 100µl) was added to one well of the plate. After incubation for 18- 24h, the cells were either lysed for luciferase assay or re-fed with complete medium containing 750µg/ml G418 for selection and colony formation assay.

Western Blotting

Western Blotting was done for protein analysis. Cells were harvested in Phosphate Buffer Saline (PBS) and centrifuged for 5-6min at 650 g at 4°C. Pellet was lysed in Lysis Buffer (50mM Tris– HCl pH 8.0, 5mM EDTA, 150mM NaCl, 0.5% NP40, 1mM PMSF, and protease inhibitors) and centrifuged for 10min at 14,000 rpm at 4°C. The supernantant was transferred

to a clean tube and protein concentration was determined using Bradford reagent. 20-30µg

protein was fractionated by SDS-PAGE and transferred to Immobilon P filters (Millipore). The

filter was blocked in PBS containing 5% non-fat dry milk and 0.1% Tween-20 for 30-60min at

room temperature. Then the filter was incubated with primary antibody diluted in blocking

solution to an appropriate concentration for 2h to overnight at 4°C. Then the filter was washed

three times in PBS with 0.1% Tween-20 for 10min each before incubating in secondary

antibody. The secondary antibody HRP IgG goat-anti-mouse or HRP IgG goat-anti-rabbit was

diluted in blocking solution and applied to the filter for 2h at room temperature. Following the

secondary antibody, filter was washed five times for 6min each with PBS and 0.1% Tween-20

and developed using either ECL-plus reagent (Amersham) or Supersignal (Pierce). Human

SIRT1 was detected using 10E4 (monoclonal) at 1:40 dilution. Acetylated E2F1 was detected

with Ack117 (polyclonal; generated in the lab) at 1:1,000 dilution, E2F1 was detected with

E2F1 KH-95 (Santa Cruz Biotechnology) at 1:3,500 dilution, phospho RB ser-795 was

detected with pRB795 polyclonal antibody (cell signaling) at 1:1,000 dilution, total RB was

detected with IF8 antibody (Santa Cruz Biotechnology) at 1:3,000 dilution.

Immunoprecipitation Assay

For immunoprecipitation assays, cells were harvested in PBS and centrifuged at 4°C for 5-6

min at 650 g. The pellet was lysed in lysis buffer (50mM Tris–HCl pH 8.0, 5mM EDTA,

150mM NaCl, 0.5% NP40, 1mM PMSF, and protease inhibitors), centrifuged for 10min at

14,000 rpm at 4°C and the supernatant was transferred to a clean microfuge tube. The

supernatant was immunoprecipitated using 1µg of E2F1-KH95 antibody and 40µl of

sepharose Protein A slurry at 4°C overnight with rotation. Next day, the beads were washed

4-5 times with lysis buffer, boiled in SDS sample buffer, fractionated by SDS-PAGE and

analyzed by Western blot.

Quantitation of DNA Synthesis

HCT116 cells were cultured in 24-well plates and EX-527 was added at the indicated concentrations for 18h. 5µCi of Methyl ^{3}H thymidine was then added for 1h to label the DNA. The cells were lysed in 0.2ml Lysis Buffer [2%SDS, 10mM EDTA pH 8.0] per well and incubated at 75°C for 20min and then vortexed for 20 seconds. 170µl of the cell lysate was applied to S/P glass fiber filters (Baxter) arranged over a sheet of aluminum foil. The filters were incubated in ice-cold 10% tricholoroacetic acid for 5-10min and then transferred to a vacuum manifold. The filters were washed with 10ml ice-cold 5% trichloroacetic acid followed by 5ml of 95% ethanol. The filters were then allowed to dry and suspended in 2ml scintillation mixture and the counts read on a liquid scintillation counter. Each sample was treated at least in triplicates or as the indicated 'n' values and the difference between each treatment was determined by calculating the p values using two-tailed student's *t*-test. p values less than 0.05 were considered significant.

Colony Formation Assay

To study the effect of constitutive overexpression of SIRT1 on colony formation, HCT116 cells were plated at 50% confluency in a 6-well plate. Each well was transfected with 1µg of one of the following plasmids: pcDNA3 vector, pcDNA3-SIRT1, pcDNA3-SIRT1 H363A deacetylase dead mutant or pcDNA3-SIRT1 K35E cytoplasmic mutant using Lipofectamine 2000. 24h after transfection, the media was changed and 750µg/ml G418 was added to the wells. The drug was constantly maintained in the wells and the colonies that were formed at the end of two weeks were visualized by staining with crystal violet.

To study the effect of 5-FU on colony formation, 100 HCT116 cells each (control and SirT1 knockdown or EX-527 treated and untreated) were plated in 6-well plates. 24h after plating, the cells were subjected to different doses of 5-FU as indicated. 24h after treatment with 5-FU, the cells were washed and re-fed with fresh medium to allow for cells to recover and form

colonies that are resistant to 5-FU. At the end of two weeks, the colonies that were formed were visualized by staining with crystal violet.

Growth Assay

To study the effect of EX-527 on proliferation of cells, 500 HCT116 cells were seeded in 6cm plates in triplicates for indicated doses of EX-527. Enough plates were prepared to study the growth over a period of 7-8 days. Three plates were harvested every 24h for each dose and cells were counted using a hemocytometer. A growth curve was plotted and standard deviation was calculated using the values obtained from the triplicate samples.

Reporter Assay

80,000 HCT116 cells were seeded in 24-well plates and transfected with 10ng cyclin D3-luc reporter plasmid, 1ng GFP, 200ng carrier DNA (salmon sperm), 5ng E2F1, 10ng CMV-LacZ, 20 or 40ng SIRT1 and 20 or 40ng SIRT1-H363A mutant as indicated. Each transfection was done in quadruplicates. Lipofectamine 2000 was used to perform the transfections and after 24-30h, the cells were lysed and the luciferase and Beta-galactosidase expression was measured. A graph was plotted that represented the luciferase values normalized with the beta-galactosidase values.

Cell Cycle and Apoptosis Assays

Cell Cycle Analysis. HCT116 cells were seeded to 90% confluency in 6cm plates and transfected with 0.5µg vector alone or 0.5µg SIRT1 using Lipofectamine 2000 reagent. 24h after transfection, cells were treated with 100 ng/ml nocodazole for 18h, and harvested by trypsinization. The pellet was washed twice with PBS and resuspended in 1ml PBS and fixed by adding 2ml ice-cold ethanol while slowly vortexing. Ethanol-fixed cells were stored at -20˚C for a minimum of 24h and then washed with PBS. The cells were then stained with primary

57

anti-SIRT1 antibody (10E4) for 3h, and secondary antimouse fluorescein isothiocyanate (FITC) for 1h with rotation at room temperature. Subsequently, the cells were stained with 50µg/ml propidium iodide (PI) and analyzed by fluorescence-activated cell sorting (FACS). Fluorescein isothiocyanate positive cells were analyzed for cell cycle distribution.

To study the effect of EX-527 and camptothecin (CPT), HCT116 cells were treated with the indicated doses of the drugs for 18h and harvested and fixed in ethanol. They were then stained with 50µg/ml PI and analyzed for apoptotic sub-2N population.

TUNEL Assay. The In Situ Cell Death Detection kit from Roche Applied Science was used to perform a TUNEL assay to study the response of HCT116 cells to 5-FU and EX-527. HCT116 cells were seeded in 10cm plates and treated with the drugs at indicated doses for 18h. Trypsin was used to harvest the cells and washed in PBS at least three times. The pellet of cells were resuspended in PBS and counted to adjust to 3 X 10^7 cells/ml in 100µl PBS. While slowly vortexing, 100µl of freshly prepared fixation solution (2% methanol-free formaldehyde in PBS pH7.4) was added to the cell suspension and the mix was incubated on ice for 15min. The suspension was centrifuged at 300 g for 10min at 4°C and washed with PBS twice and finally resuspended in 0.5ml PBS. To this, 5ml of ice-cold 70% ethanol was added and incubated overnight. Next day, cells were washed twice with PBS and resuspended in 50µl TUNEL reaction mixture (50µl of TdT or Terminal deoxynucleotidyl Transferase with 450µl F-dUTP label solution as suppled with the kit). At the same time, a negative control was prepared in which the TUNEL reaction mix only consisted of label solution and a positive control in which the fixed cells had been treated with micrococcal nuclease or DNase I for 10min at room temperature to induce DNA strand breaks. The positive control was also resuspended in 50µl of TUNEL reaction mix. All samples were incubated for 1h at 37°C in a humidified incubator in dark. Cells were washed twice with PBS and resuspended in 1ml of PBS and analyzed by FACS for green fluorescence positive cells.

Immunohistochemical Staining

For TMA staining, the Colon Tumor Microarray was developed from patient samples collected at the Moffitt Cancer Center, Tampa, FL. This tissue micro array includes over 100 colon tumor tissue samples and normal colon tissues. The tissue micro array was stained for SIRT1 using the 10E4 monoclonal antibody by the Histopathology Core at the Moffitt Cancer Center. The tissue microarray slide was processed using a Ventana Discovery XT automated system (Ventana Medical Systems) as per the manufacturer's protocol with proprietary reagents. Slides were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was performed using Cell Conditioning Solution (Ventana). The 10E4 hybridoma supernatant was used at 1:150 dilution in Dako antibody diluent and incubated for 60min. The Ventana Universal Secondary Antibody was used for 32min at 37°C. The detection system used was the Ventana DABMap kit. Slides were counterstained with hematoxylin and scanned by the Analytical Microscopy Core. The SIRT1 staining level of each tumor tissue sample and the normal control tissues was examined by three individuals and scored from 0 to 3 based on the intensity of the stain.

For Staining Apc^{Min} Mouse Tissue Slides, intestines were harvested from the desired genotypes and fixed in 10% buffered formalin overnight followed by paraffin embedding and sectioning. The tissue slides were deparaffinized by subjecting to three changes of xylene for 10min each followed by rehydration using decreasing gradient of ethanol (100%; 95%; 70%; 50%; 30%; water). Slides were incubated in freshly prepared Sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0) for 30min at 95°C and then cooled for 20min at room temperature. Then slides were washed in two changes of 1X PBS and incubated in 0.3% H_2O_2 for 15min at room temperature to quench endogenous peroxidase activity followed by three washes with 1X PBS. The ABC staining system for Rabbit antibody (Santa Cruz Biotechnology) was used for staining. Briefly, the tissue sections were blocked in 1.5%

Normal Goat Serum for 1h followed by incubation in primary antibody, polyclonal anti-Sir2 antibody (Upstate # 07-131) at 1:500 dilution for 16h at 4°C. Then slides were washed in PBS and incubated in biotinylated secondary antibody for 30min at room temperature followed by incubation with the AB enzyme reagent and developed in DAB chromogen.

Animal Experiments

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Florida.

Tumor Xenograft Experiments. For the tumor xenograft assays, 7 to 8 weeks old Athymic-NCr-nu female mice were inoculated subcutaneously on both flanks with 8×10^6 HCT116 control or *SIRT1* siRNA cells. To study the effect of SIRT1 overexpression, 8×10^6 HCT116 control or lenti-SIRT1 cell were injected on both flanks of the nude mice and SIRT1 expression was induced by feeding the mice with a diet containing 500mg/kg doxycycline to induce the lentiviral expression of SIRT1.Tumors were measured every two days with digital calipers and the tumor volumes were calculated using the formula [(average of two diameters)/2]^3 * 0.5236. Paired student's *t*-test was used for statistical analysis and calculation of the p value where $p < 0.05$ was considered a significant value for difference between tumor volumes. The tumor xenograft assay performed with A549 *SIRT1* siRNA and control cell lines followed the same protocol as described above for the HCT116 cells.

Animal Breeding. Apc^{Min} mouse was purchased from The Jackson Laboratory as a male C57Bl/6J heterozygous for the *Min* mutation in the *Apc* gene (Δ850). The *Min* mutation indicates a point mutation in one allele of the *Apc* gene such that codon 850 is converted from a leucine to a STOP codon and the resulting protein is truncated. The heterozygous genotype was propagated by breeding with wild-type C57Bl/6 females and a colony was established.

Apc^{Min} null mice were not viable. The Apc^{Min} heterozygous mice develop spontaneous intestinal polyps by 60 days of age upon losing the wild type allele of the *Apc* gene thereby mimicking the mutation that causes Familial Adenomatous Polyposis (FAP) in humans. Genotyping was done using primers that recognize the mutant allele of the *Apc* gene. Min Forward primer: 5' TGA GAA AGA CAG AAG TTA 3' and Min Reverse primer: 5' TTC CAC TTT GGC ATA AGG C 3'. PCR conditions: 94°C for 3min; 35 cycles of 94°C for 30s, 55°C for 1min, 72°C for 30s; followed by 72°C for 7min. The amplified mutant allele appears at 340 bp.

The Sirt1 heterozygous mice in 129sv background were kindly provided by Dr. Fred Alt and Dr. Toren Finkel. In these heterozygous mice, one allele of the *Sirt1* gene contains a targeted LoxP-pgk-NeoR selection cassette, the presence of which reduces the total Sirt1 protein level in heterozygous MEFs as observed by Western blotting with anti-Sir2 antibody. Genotyping for these mice were done using three primers in the same PCR reaction to simultaneously detect the mutant allele (800 bp) and the wild type allele (500bp) of Sirt1. Forward Primer KO-F 5' CTTGCACTTCAAGGGACCAA 3' and two reverse primers SKO-R1 5' GTATACCCACCACATCTGAG 3' and SKO-R2 5' CTACCACTCCTGGCTACCAA 3'. PCR conditions: 95°C for 5min; 35 cycles of 95°C for 40s, 54°C for 50s, 72°C for 90s; followed by 72°C for 10min.

To study the effect of Sirt1 on polyp formation in Apc^{Min} mice, Sirt1 heterozygous mice were bred with Apc^{Min} mice and the progenies were genotyped. Apc^{Min} progenies that were wild type or heterozygous for Sirt1 were selected and sacrificed at 6 months of age and the intestines were harvested and fixed in formalin. This was followed by paraffin embedding, sectioning and H&E staining. Polyps were counted and noted. The difference in the number of polyps between the two genotypes was calculated by using student's *t*-test where $p < 0.05$ is significant.

To study the effect of SIRT1 on lung tumorigenesis, a lung tumor mouse model was purchased from the Mouse Repository (Mouse Models of Human Cancers Consortium). The K-rasLA2 mouse strain (Johnson et al., 2001) contains a latent *Kras* allele that can be activated by spontaneous mitotic recombination resulting in an active *KrasG12D* mutant allele. As a result, mice heterozygous for this allele develop tumors mainly in the lungs and their mean survival age is around 200 days. Male Kras mutant was obtained and bred to female C57Bl/6 mice and a colony was established. Mice were genotyped by using three primers in the same PCR reaction. One common forward primer was used and two reverse primers that could recognize either the wild type allele or the mutant allele (K001 forward: 5' TGCACAGCTTAGTGAGACCC 3'; K002 reverse wild type: GACTGCTCTCTTTCACCTCC 3'; K003 reverse mutant: 5' GGAGCAAAGCTGCTATTGGC 3').

For the experiment, Sirt1 heterozygous mice were bred with K-rasLA2 heterozygous mice and the K-rasLA2 progenies that were wild type or heterozygous for Sirt1 were selected (*KrasG12D;Sirt1+/+* or *KrasG12D;Sirt1+/-*). PCR conditions: 94˚C for 3min; 35 cycles of 94˚C for 1min, 60˚C for 2min, 72˚C for 1min followed by 72˚C for 3min.

To study the effect of Sirt1 on lung tumor nodule formation, 12 mice from each genotype were sacrificed at 20 weeks of age. The lungs were harvested and nodules on all five lobes were counted. The lungs were then insufflated and fixed in formalin and paraffin embedded, sectioned and stained for Sirt1 levels using anti-Sir2 antibody. The difference between the numbers of tumor nodules from the two genotypes were calculated using the student's *t*-test where $p < 0.05$ is significant.

To study the effect of Sirt1 on survival of the K-rasLA2 mice, 36 *KrasG12D;Sirt1+/+* and 24 *KrasG12D;Sirt1+/-* were observed and their dates of birth and death were recorded. A Kaplan-Meier Curve was plotted and the statistical significance of survival between the two groups was tested by calculation of p value using the chi-square test.

Generation of MEFs. To generate Sirt1 wild type, Sirt1 heterozygous and Sirt1 homozygous null MEFs, Sirt1 heterozygous male was bred to Sirt1 heterozygous female and embryos were harvested. The protocol was modified from Boris Greber. Pregnant females were sacrificed at 13dpc by cervical dislocation and the oviducts (left and right) were removed and rinsed with 70% ethanol. Then the oviducts were washed twice with PBS to remove any traces of ethanol. Working in a sterile environment, embryos were removed one at a time from the oviduct and placed in different petri-dishes to avoid cross-contamination. Each embryo was separated from the surrounding membrane and the placenta and minced such that it was pipettable. Using a small amount of trypsin, each embryo was transferred to a separate microfuge tube and placed in a mixer/shaker at 600-800 rpm for 30min at 37°C to allow the contents to break up. The contents of the microfuge tube were then mixed with 5ml of complete medium (DMEM+10% FBS) and the debris was allowed to settle. Supernatant was transferred to a separate tube and spun to obtain a cell pellet. The cell pellet was re-suspended in culture medium and plated in a culture dish followed by overnight incubation at 37°C in an incubator. Next day, the cells obtained from each embryo were trypsinized and half the cells were frozen as Passage-P0 and the other half were collected for Western analysis to detect the expression of Sirt1.

Chapter Three

Results

SIRT1 Knock Down Promotes Growth of Colon Tumor Xenograft

Previous research and results have suggested that SIRT1 exhibits pleiotropic functions in cell

culture experiments, i.e., anti-apoptotic as well as anti-proliferative. To further test the role of

endogenous SIRT1 in tumor formation, HCT116 colon carcinoma cells were stably infected

with retrovirus vector (pSuperior) expressing control or *SIRT1* shRNA. Polyclonal cell lines

were generated from pooled colonies and confirmed for the knock down of SIRT1 (Figure 6).

To test their ability to form tumors in xenograft assay, cells were inoculated subcutaneously

on the dorsal flanks of athymic nude mice. Each animal received both control and knock down

cell lines to facilitate comparison under similar conditions (Figure 6).
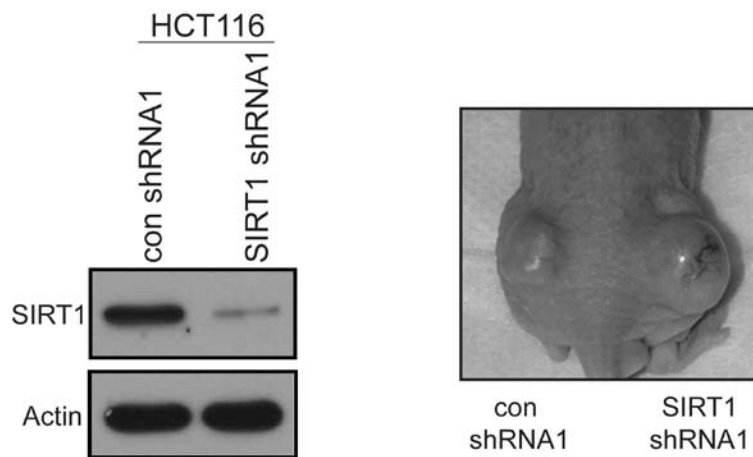
Figure 6: Endogenous SIRT1 Expression Limits Tumorigenicity. A retrovirus vector
pSuperior expressing control or *SIRT1* shRNA (SIRT1 shRNA1) was used to infect HCT116
cells, and the colonies were pooled to generate a polyclonal SIRT1 knock down cell line. The
expression of SIRT1 in the control and knock down cells was determined by Western blot.
Cells were injected subcutaneously in the dorsal flanks of athymic nude mice, and tumor
formation in a representative animal is shown.

The results showed that tumors formed from SIRT1 knock down cells were 2- to 3-fold larger than control cells inoculated in the same animal (*n*=10, *p*=0.0007 using student's *t*-test where *p* < 0.05 is significant) (Figure 7).
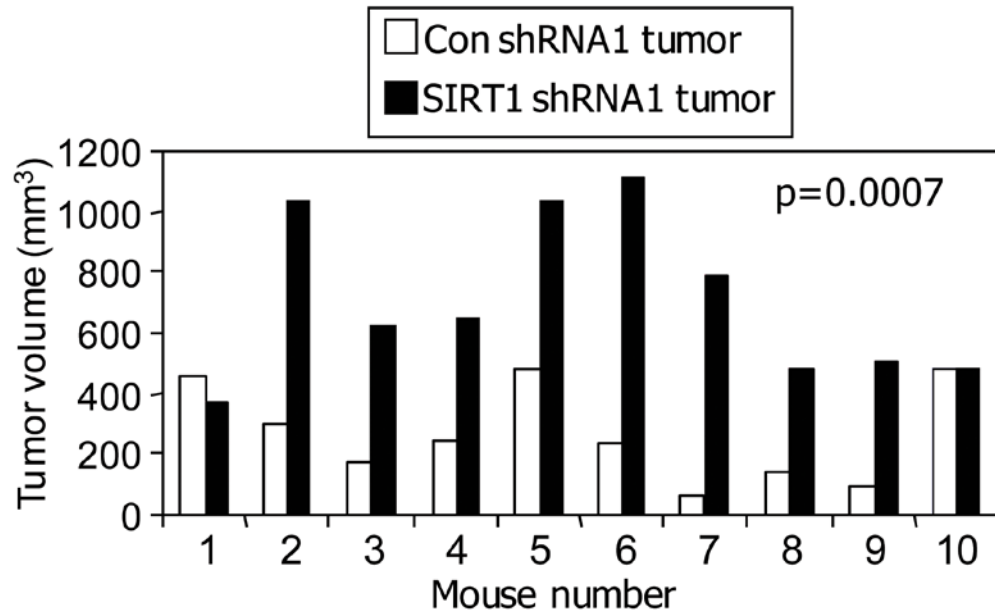

Figure 7: SIRT1 Knock Down Promotes Tumorigenicity Tumors arising from the SIRT1 control and knock down cell lines were measured after 14–21 days and their volumes were calculated (*n*=10, *p*=0.0007 as calculated using paired student's *t*- test where *p*<0.05 is statistically significant).

To ensure that the tumors arising from the SIRT1 knock down cell line had maintained low SIRT1 levels, tumors from five random mice were harvested, homogenized and lysed in Lysis Buffer. The expression of SIRT1 in these tumors was tested by Western Analysis (Figure 8).
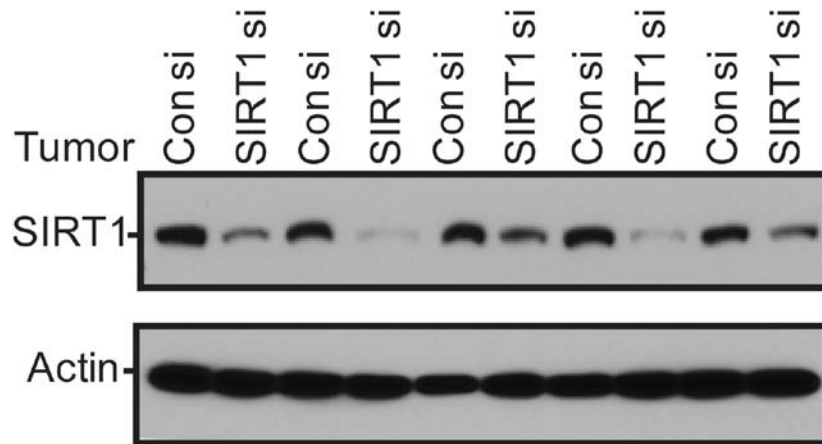
Figure 8: Low SIRT1 Levels are Maintained in Tumors Arising from SIRT1 Knock Down Cell Lines: Five mice were randomly selected and the tumors from each flank were harvested, homogenized and lysed. SIRT1 levels were detected using anti-Sir2 antibody (Upstate Biotech).

To rule out off-target effects of SIRT1 shRNA1, a separate pair of HCT116 control and knock down cell lines was generated using completely different control shRNA and *SIRT1* shRNA sequences and expression vectors obtained from the OpenBiosystems shRNA library. The xenograft experiment was repeated and it was observed that SIRT1 knock down cells formed significantly larger tumors than control cells (n =13, p =0.01 using student's t test, where p <0.05 is significant (Figure 9a and 9b) thereby confirming the results obtained from SIRT1 shRNA1. Together, these results suggest that endogenous SIRT1 in HCT116 cells inhibits the growth of tumor xenografts.
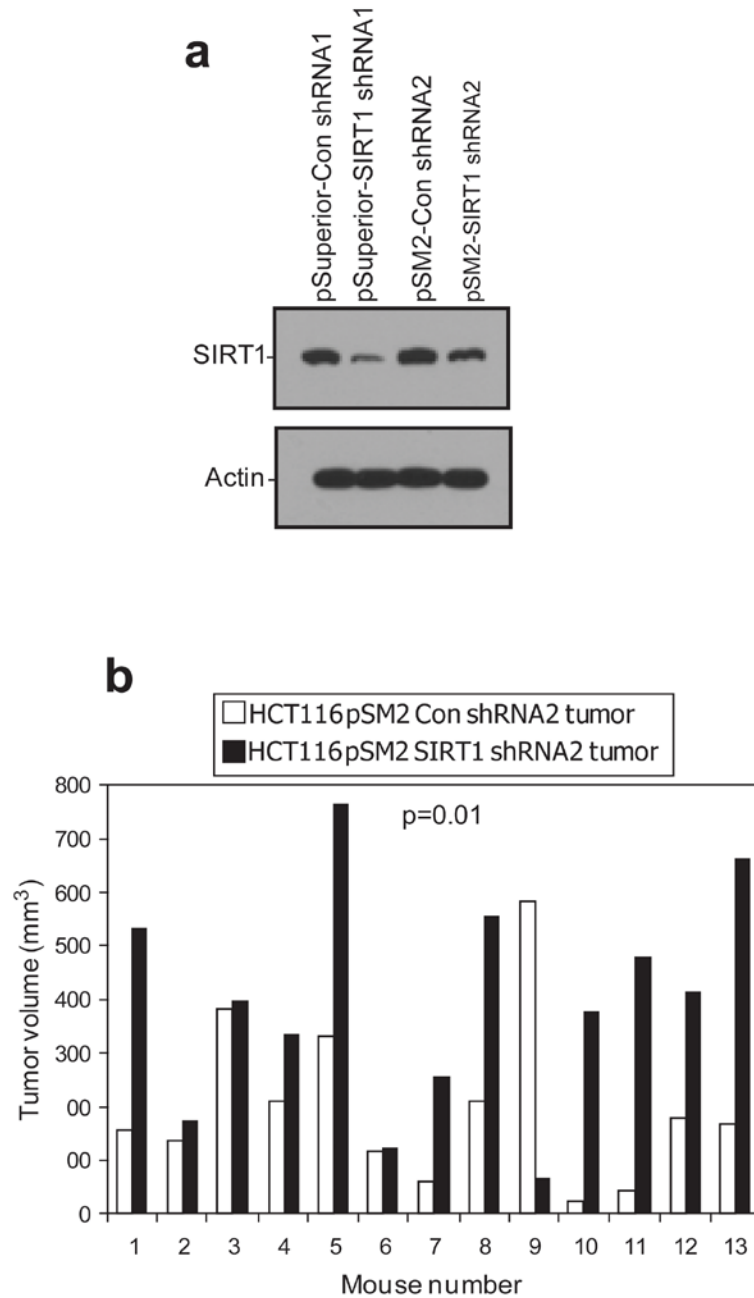
Figure 9: Knock Down of SIRT1 Increases Tumorigenicity. (a) pSM2-SIRT1shRNA2
vector was used to generate a second HCT116 knock down cell line. SIRT1 knock down
efficiency was compared to cells with pSuperior-SirT1shRNA1 by western blot.
(b) HCT116 SIRT1 knock down and control cells were injected subcutaneously in the dorsal
flanks of athymic nude mice and tumor volumes were measured. Tumors formed from SIRT1
knock down cells were larger than those from control cells (n=13 and p=0.01).

The role of SIRT1 in suppressing tumor growth was not limited to colon tumors. A xenograft

assay using A549 lung tumor cells with SIRT1 knock down also resulted in accelerated tumor

growth (Figure 10).
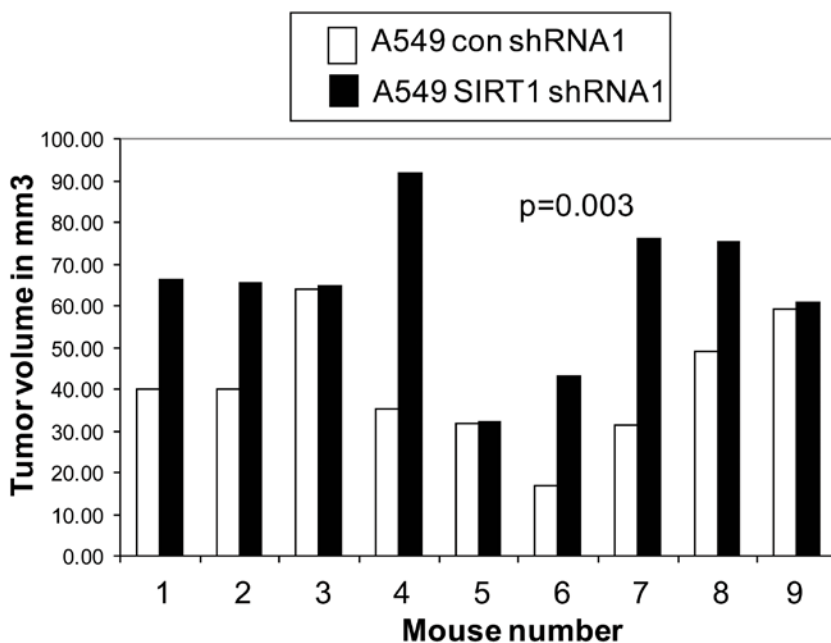
Figure 10: Knock Down of SIRT1 Increases Tumorigenicity in Lung Cancer Cell Line.
Eight million A549 control and SIRT1 knock down cells were injected subcutaneously in the
dorsal flanks of athymic nude mice and tumor volumes were measured after 14-21 days.
Tumors formed by SIRT1 knock down cells were larger than those from control cells (n=9 and
p=0.003).

SIRT1 Overexpression Suppresses the Growth of Colon Tumor Xenograft

The above results suggest that expression of endogenous SIRT1 partially suppresses tumor

formation. To further test this hypothesis, HCT116 cells were stably infected with a

tetracycline-inducible lentivirus vector expressing SIRT1. In this cell line, SIRT1 expression

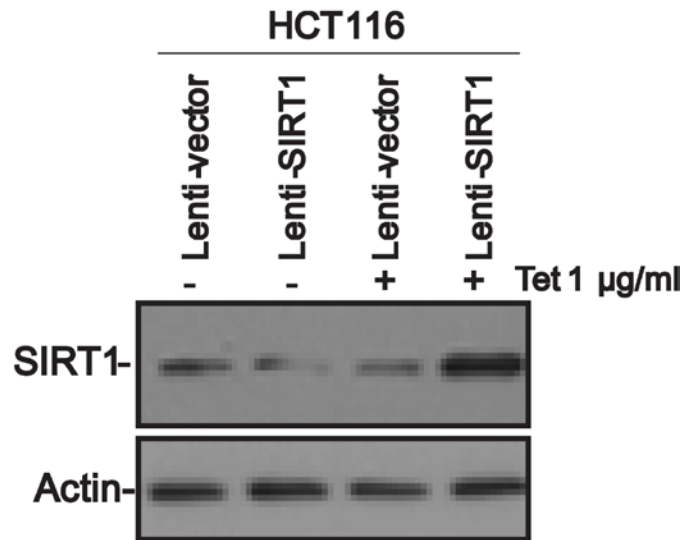could be induced to almost 4-fold above endogenous level by 1μg/ml tetracycline (Figure 11).

Figure 11: Generation of HCT116 Inducible SIRT1 Cell Lines. HCT116 cells were infected with a tetracycline-inducible lentivirus vector expressing SIRT1 or control vector. SIRT1 expression was induced by 1µg/ml Tetracycline within 24h. The levels of SIRT1 were detected by Western blot.

To test the effect of SIRT1 overexpression on tumor growth, nude mice were injected on either side of their flanks with lenti-vector- and lenti-SIRT1-infected HCT116 cells. These mice were kept on a diet containing doxycycline to induce SIRT1 expression. The results showed that, in contrast to the phenotype of SIRT1 knock down, overexpression of SIRT1 significantly reduced the tumorigenicity of HCT116 cells (Figure 12). It is noteworthy that in a significant fraction of cases (8 of 14), HCT116-lenti-SIRT1 cells gave rise to extremely small or no tumors. This suggests that, besides inhibiting cell proliferation, overexpression of SIRT1 may suppress the initial establishment of tumors in this setting.
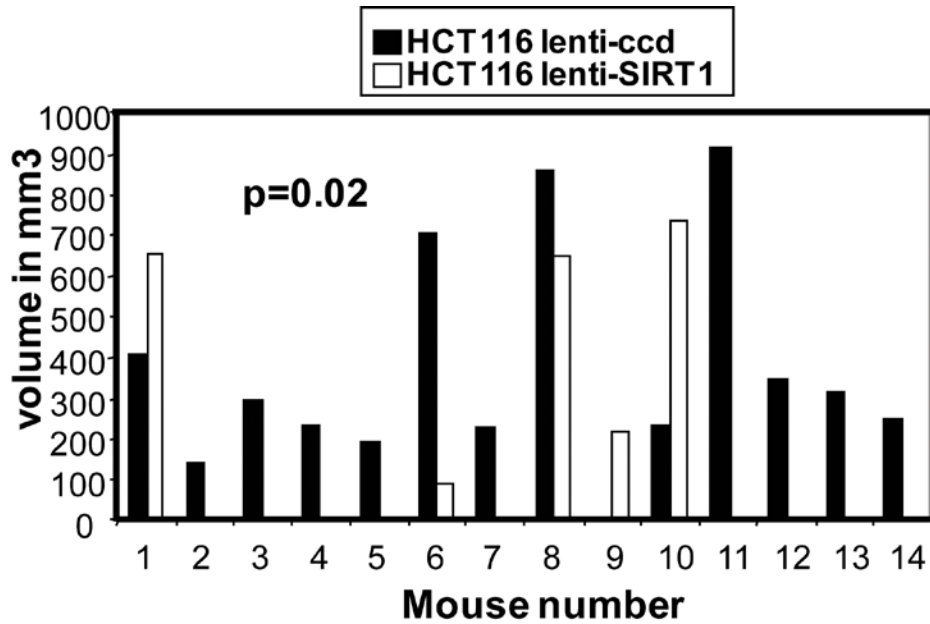
Figure 12: SIRT1 Overexpression Suppresses the Growth of Colon Tumor Xenograft:
Athymic nude mice were injected on the dorsal flanks with eight million HCT116-lenti-vector
and HCT116-lenti-SIRT1 cells. The mice were continuously fed with chow containing
doxycycline (500 mg/kg). Tumors were measured and the graph indicates the tumor volumes
generated by the control *versus* the SIRT1-overexpressing cell line (n=14, p=0.02 as
calculated with paired student's *t*- test).

To ensure that SIRT1 was being induced from the Lentivirus by doxycycline, pairs of tumors
from four random mice were harvested, homogenized and lysed in Lysis Buffer and SIRT1
levels were tested by Western Analysis (Figure 13). SIRT1 overexpression was found to be
almost four fold in the tumors arising from the Lenti-SIRT1 cell line as compared to the tumors
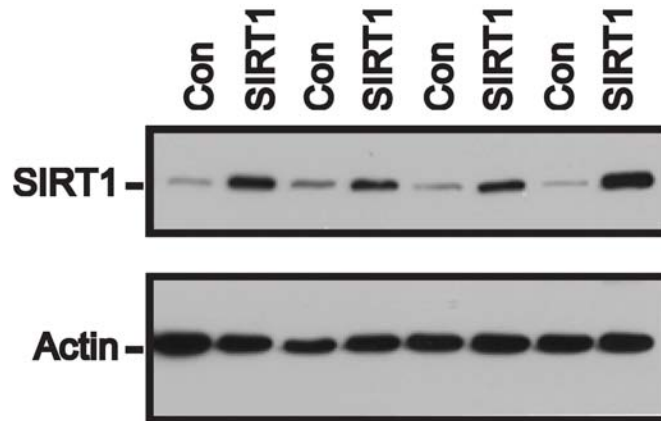arising from the Lenti-vector cell line.

Figure 13: Doxycycline Induces SIRT1 Expression in Tumor Xenograft Assays. Mice were continuously fed with chow containing doxycycline at a concentration of 500mg/kg. Four mice were randomly selected and the tumors were harvested and lysed. SIRT1 induction was detected by Western analysis using anti-Sir2 antibody.

Inactivation of SIRT1 Stimulates Tumor Cell Proliferation

To further test whether physiological levels of endogenous SIRT1 plays a role in regulating cell proliferation, cells were treated with SIRT1-specific inhibitor EX-527. This compound has significantly improved potency and specificity against SIRT1 as compared to nicotinamide (Solomon et al., 2006). When HCT116 cells were cultured in 0.1% serum, addition of EX-527 caused a 90% increase in cell number after 7 days (Figure14).
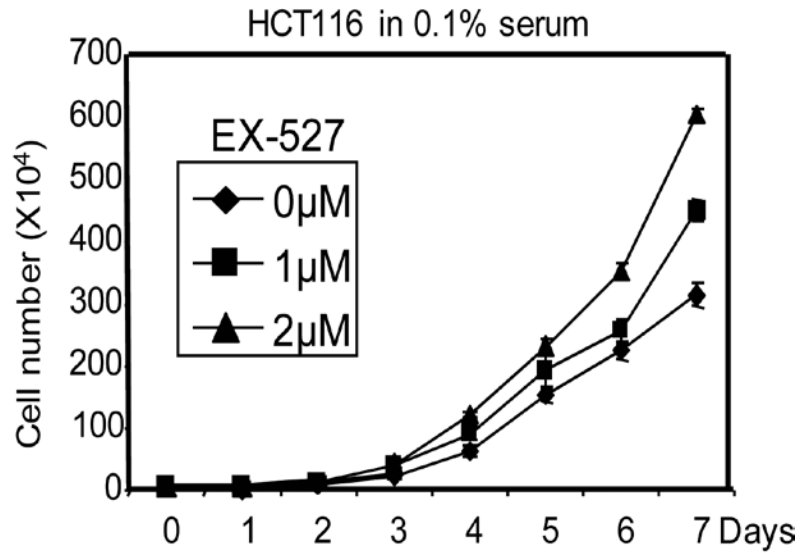
Figure 14: Inhibition of SIRT1 Stimulates Cell Proliferation upon Nutrient Deprivation (Growth Assay). HCT116 cells cultured in 0.1% serum were maintained in different concentrations of SIRT1-specific inhibitor EX-527, and cell number was determined at the indicated time points. *Error bars* represent mean $\pm$S.D. (n=3).

In the presence of 10% serum, EX-527 did not change cell number in long term culture (Figure15). This result shows that, upon growth factor deprivation, SIRT1 functions as a significant regulator of cell proliferation.
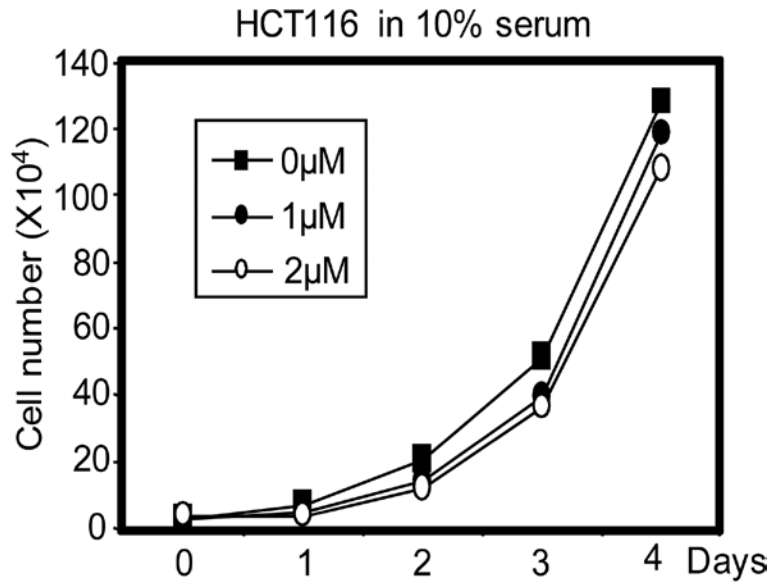
Figure 15: Inhibition of SIRT1 Does Not Affect Cell Proliferation in Normal Growth Conditions (Growth Assay). HCT116 cells cultured in 10% serum were maintained in different concentrations of EX-527 and cell number was determined at indicated time points.

DNA replication was analyzed by [3H] thymidine incorporation assay to accurately measure changes in cell proliferation rate. The results showed that treatment with EX-527 stimulated DNA replication by up to 30% in low serum (Figure 16).
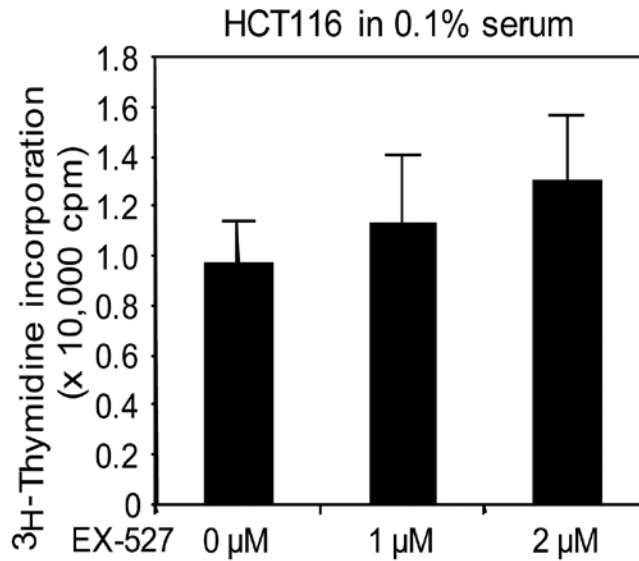
Figure 16: Inhibition of SIRT1 Stimulates Cell Proliferation upon Nutrient Deprivation.
HCT116 cells cultured in 0.1% serum were treated with EX-527 for 18h. DNA replication was measured by [3H] thymidine incorporation assay. *Error bars* represent mean $\pm$S.D. (n=14) and p=0.03 as calculated with student's *t* test where $p<0.05$ is statistically significant.

Despite the moderate magnitude of enhancement, the growth-stimulating effect of EX-527 was statistically significant (p =0.03 using student's *t* test) and highly reproducible in replicate assays. The moderate but sustained increase in cell proliferation is likely responsible for the large increase in cell population. However, when cells were cultured in 10% serum that supports optimal growth, EX-527 treatment did not stimulate further DNA synthesis thereby underlining the importance of SIRT1 as a growth regulator in stressed conditions (Figure 17).
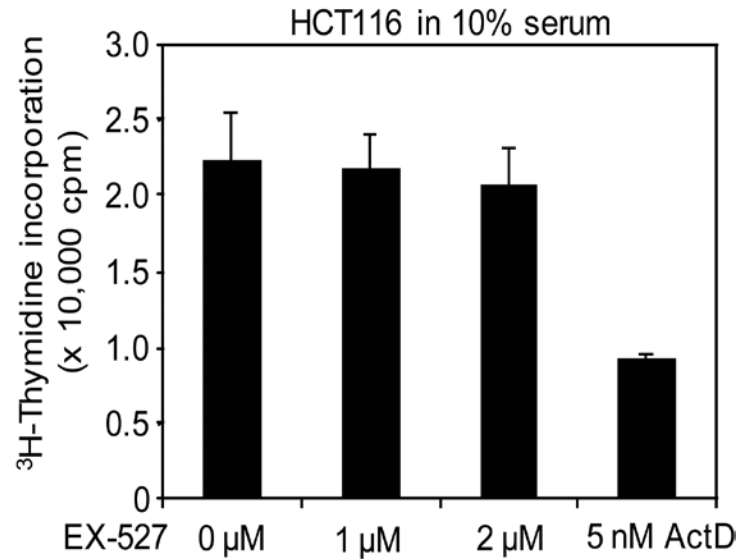
Figure 17: Inhibition of SIRT1 Does Not Affect Cell Proliferation in Normal Growth Conditions. HCT116 cells cultured in 10% serum were treated with SIRT1-specific inhibitor EX-527 for 18h. DNA replication was measured by [3H] thymidine incorporation assay. Actinomycin D, a known inhibitor of cell proliferation was used as a control. *Error bars* represent mean $\pm$ S.D (n=6). p=0.38 between 2μM EX-527 treated cells and control cells suggesting no significant difference.

Importantly, inhibition of SIRT1 also stimulated the proliferation of A549 (lung tumor) and U2OS (osteosarcoma) cells suggesting that its growth-suppressive effect is not limited to colon cancer cells (Figure 18a and b).
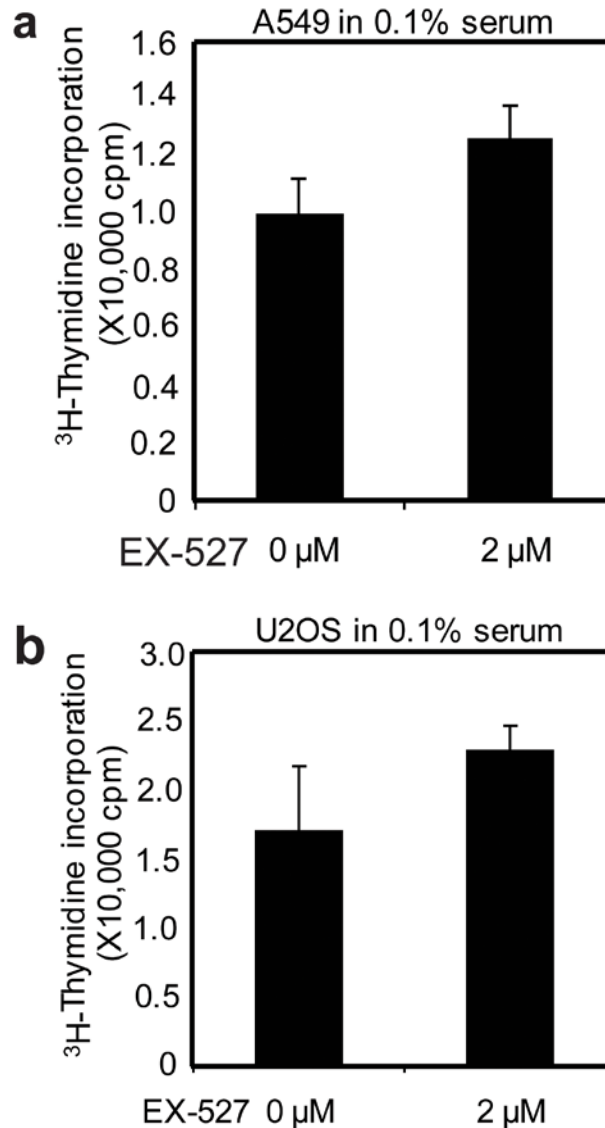
Figure 18: Inhibition of SIRT1 Stimulates Cell Proliferation in Different Cell Lines. A549 and U2OS cells cultured in 0.1% serum were treated with EX-527 for 18h. DNA replication was measured by [3H] thymidine incorporation assay. *Error bars* represent mean ± S.D. (n=6). p=0.005 for figure 18a and p=0.02 for figure 18b suggesting a significant increase in proliferation of cells treated with EX-527 as compared to control cells.

To demonstrate that the growth-stimulatory effect of EX-527 is due to inhibition of SIRT1, mouse embryonic fibroblasts (MEFs) derived from Sirt1 null mice were analyzed. Initial experiments revealed that late-passage Sirt1 null and wild type MEFs were significantly different in their proliferation rate and morphology thereby making them unsuitable for such

analysis. Therefore, we generated low passage (P4) *Sirt1-/-* and *Sirt1+/+* MEFs from

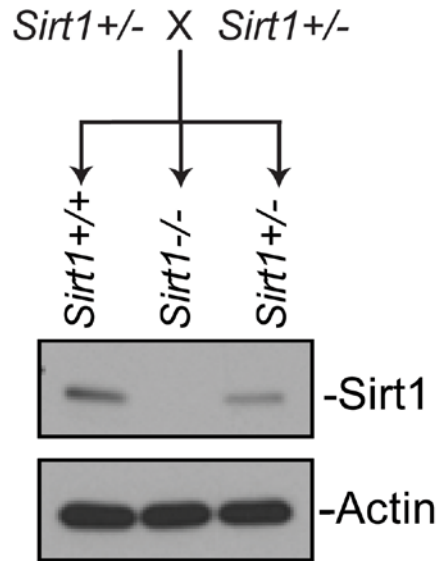littermate embryos obtained by breeding *Sirt1 +/-* mice (described in Materials and Methods).

Figure 19: Expression of Sirt1 in MEFs. Sirt1 heterozygous mice were bred and the
embryos were harvested 13dpc to generate MEFs from littermates. Sirt1 levels were
determined by Western Blotting using anti-Sir2 antibody (Upstate Biotechnology). Figure
demonstrates the absence of Sirt1 expression in *Sirt1-/-* MEFs and reduced expression in
Sirt1+/- MEFs as compared to the *SirT1+/+* MEFs.

Early passage *Sirt1-/-* MEFs clearly showed more robust growth in culture, and a significantly

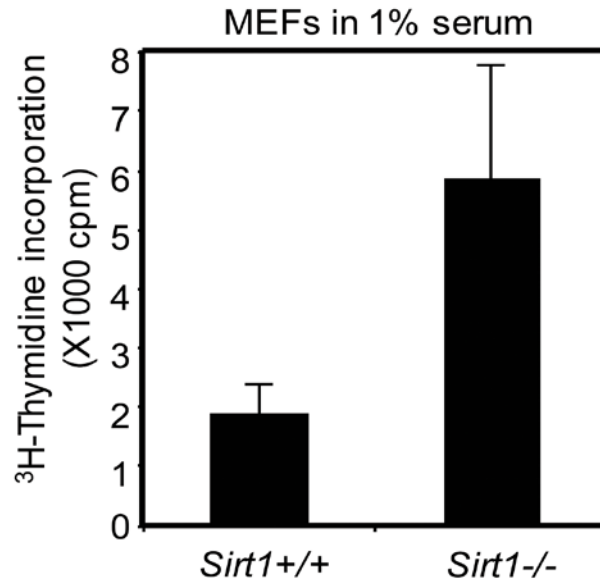higher rate of DNA synthesis (~150%) when compared with *Sirt1+/+* MEF (Figure 20).

Figure 20: Absence of Sirt1 Stimulates Cell Proliferation. Early passage (P4) MEFs from *Sirt1+/+* and *Sirt1-/-* mouse embryos were tested for DNA synthesis rate by [3H] thymidine incorporation. An identical number of cells from each genotype were plated prior to 18h culture in 1% serum and 1h metabolic labeling. *Error bars* represent mean $\pm$ S.D. (n= 6) p=0.00001.

Treatment with EX-527 also induced a statistically significant 30% increase in DNA synthesis in *Sirt1+/+* MEF (Figure 21a). Importantly, no growth stimulation by EX-527 was observed in the Sirt1 null MEF (Figure 21b). These results demonstrate that SIRT1 is a suppressor of cell proliferation and that the growth-promoting activity of EX-527 compound is due to specific inhibition of its intended target SIRT1.
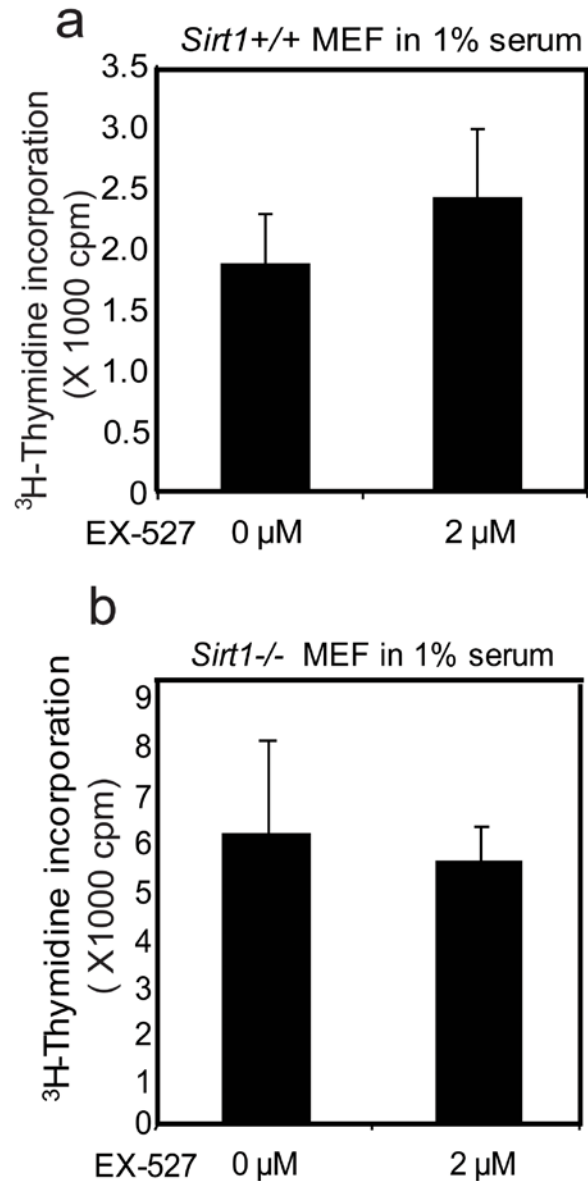
Figure 21: Increased Cell Proliferation is a Result of Sirt1 Inhibition. Early passage (P4) MEFs from *Sirt1+/+* and *Sirt1-/-* mouse embryos were tested for DNA synthesis rate by [3H] thymidine incorporation. Identical numbers of cells from each genotype were plated prior to 18h culture in 1% serum and 1h metabolic labeling. *Error bars* represent mean ± S.D (n=12). p=0.017 in figure 21a which indicates a significant increase in proliferation of *Sirt1+/+* MEFs cells treated with 2μM EX-527 as compared to control. However, no stimulation of proliferation is observed in the *Sirt1-/-* cell line when treated with EX-527 as observed in figure 21b.

SIRT1 Inhibits Cell Proliferation

To further test the potential of SIRT1 in suppressing cell proliferation, HCT116 cells were

used in a colony formation assay. Expression of SIRT1 from pcDNA3 vector significantly

reduced the number of G418-resistant colonies formed after transfection (Figure 22). In

contrast, SIRT1 NLS mutant (K35E) that failed to enter the nucleus, or deacetylase-inactive

mutant (H363A) did not suppress colony formation. Additionally, immunofluorescence staining

of pooled HCT116 G418-resistant colonies obtained from stably transfecting SIRT1 failed to

detect SIRT1 overexpression (data not shown) suggesting that continuous overexpression of

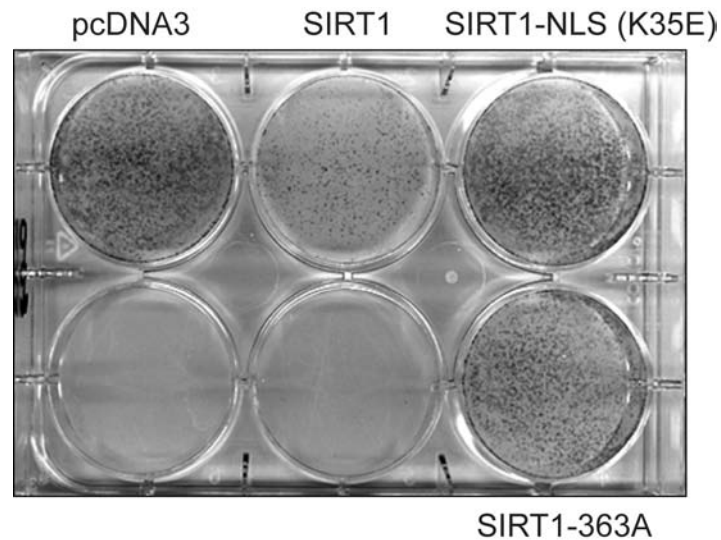SIRT1 is detrimental to cell survival.

Figure 22: SIRT1 Inhibits Cell Proliferation (Colony Formation Assay). HCT116 cells
were seeded in a 6-well plate and transfected with either pcDNA3 vector, SIRT1, SIRT1-K35E
NLS mutant, or SIRT1-H363A deacetylase mutant. Transfected cells were subjected to
selection with 750µg/ml G418, and the colonies were stained with Crystal Violet after 2
weeks.

Fluorescence-activated cell sorting analysis of HCT116 cells transiently transfected with

SIRT1 showed that SIRT1 overexpression induced efficient G1 cell cycle arrest (Figure 23)

which is consistent with the lack of outgrowth of SIRT1 overexpressing cells after long term
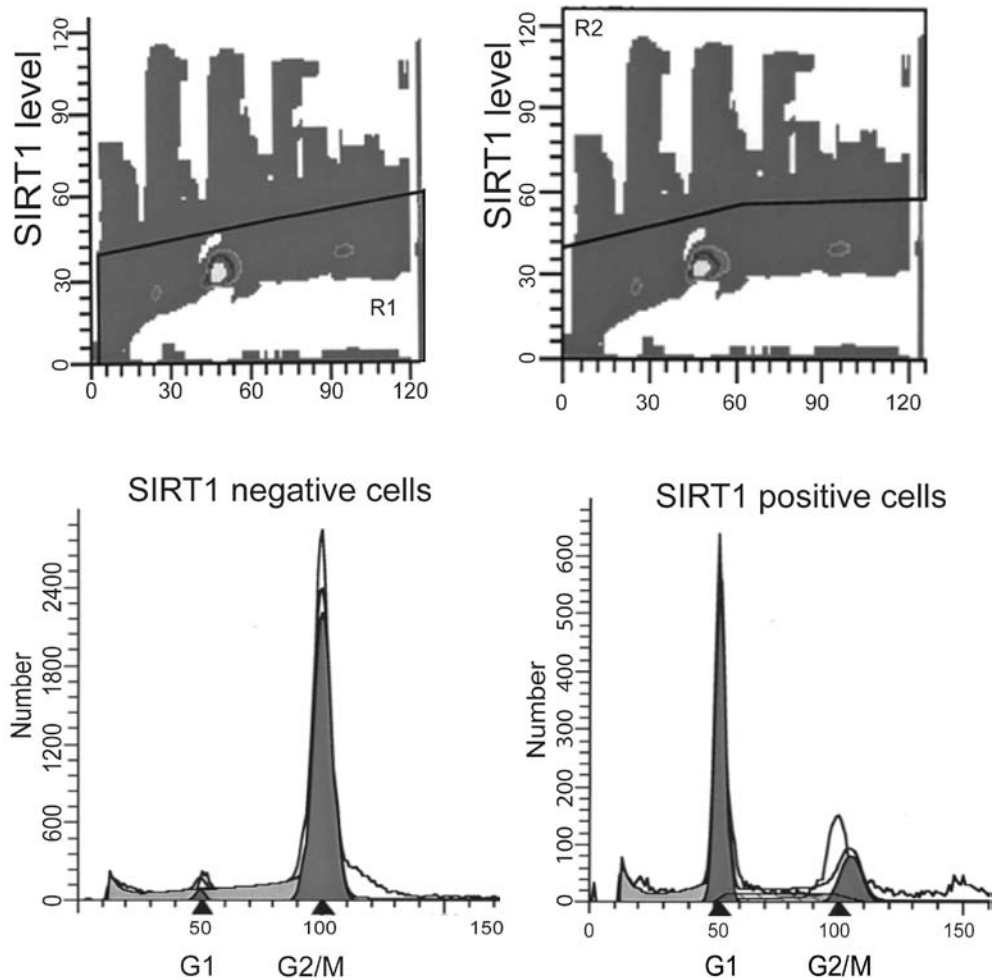
culture.

Figure 23: Inhibition of Cell Proliferation by SIRT1 (FACS Analysis). HCT116 cells were transfected with SIRT1 for 24h, treated with nocodazole for 18h, and stained using SIRT1 antibody. SIRT1 negative cells (Gate R1 in the upper left panel and cell cycle distribution in the lower left panel) and SIRT1 positive cells (Gate R2 in the upper right panel and cell cycle distribution in the lower right panel) were analyzed.

SIRT1-mediated regulation of cell proliferation is expected to be associated with changes in cell cycle machinery. To this end, hyperphosphorylation at ser-795 residue on the retinoblastoma protein (RB) was reproducibly detected after EX-527 treatment of serum-starved HCT116 cells suggesting a cell cycle inhibitory effect of SIRT1 (Figure 24). In contrast, cells cultured in 10% serum already exhibited higher basal phosphorylation at ser-

795 residue that was not stimulated any further by EX-527 (Figure 24). These results further

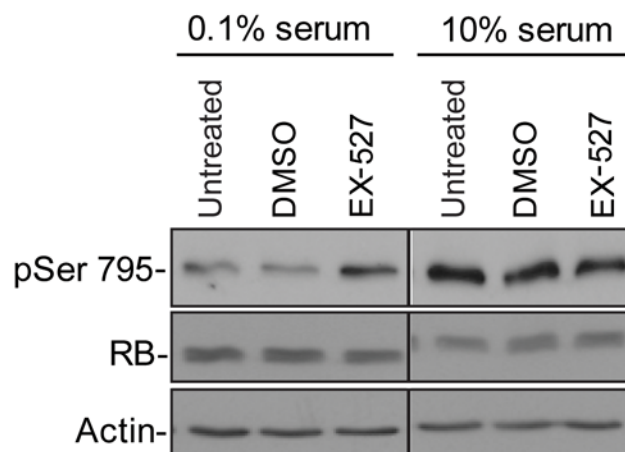confirm that SIRT1 in tumor cells may act as a suppressor of cell proliferation during stress.

Figure 24: SIRT1 Inhibition is Associated with Increased RB Phosphorylation. HCT116
cells treated with 2µM EX-527 were analyzed for phosphorylation level of RB at ser-795 by
Western blot (anti-phospho serine 795, Cell Signaling), followed by re-probing with RB
antibody (IF8, Santa Cruz Biotechnology).

Among the various SIRT1 substrates, E2F1 is a likely mediator of the growth suppressive

effect of SIRT1. Previous results from our laboratory have shown that SIRT1 binds to E2F1

and inhibits its transcriptional activity. It was shown that cell cycle arrest caused by

overexpression of SIRT1 could be rescued by co-expressing E2F1 (Wang et al., 2006). We

confirmed that in a reporter assay, expression of SIRT1 inhibited E2F1-mediated activation of

cyclin D3 promoter in a deacetylase dependent fashion (Figure 25).
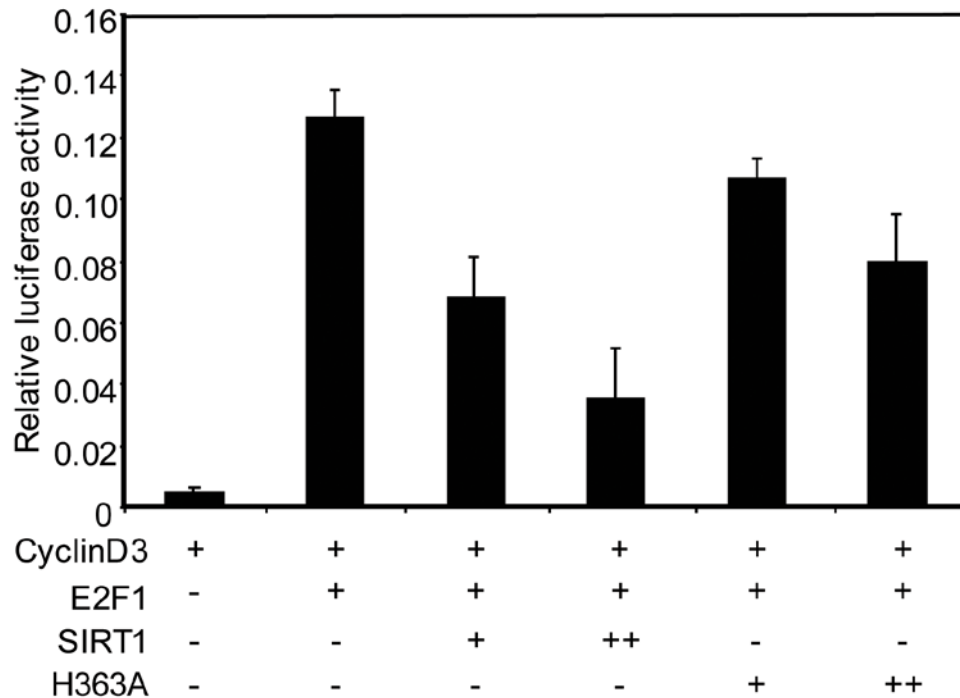
Figure 25: SIRT1 Inhibits E2F1-Mediated Activation of Cyclin D3 Promoter. H1299 cells were transiently transfected with cyclin D3 promoter-luciferase, E2F1, SIRT1, and SIRT1-H363A mutant. Promoter activity was measured by luciferase assay and normalized to cotransfected CMV-lacZ level. *Error bars* represent mean $\pm$S.D. (n=4).

Using an E2F1 K117 acetylation-specific antibody generated in our laboratory (refer to Materials and Methods), we found that E2F1 acetylation induced by the coactivator CREB-binding protein (CBP) was strongly inhibited by SIRT1. The SIRT1–H363A catalytic mutant was partially defective for E2F1 deacetylation in this assay, possibly due to residual activity when expressed at high levels (Figure 26).
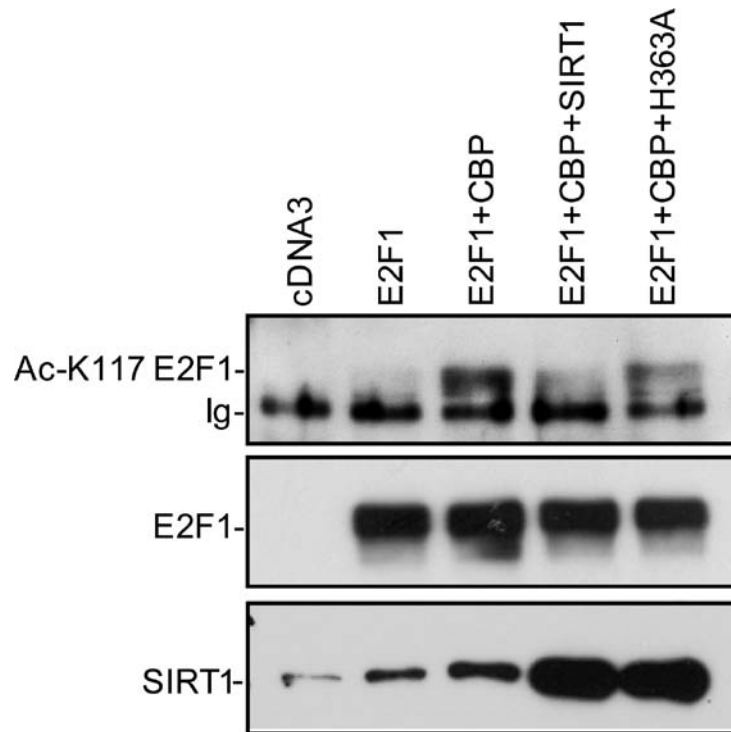
Figure 26: SIRT1 Deacetylates E2F1. 293T cells were transiently transfected with E2F1, HA tagged CBP (CREB-binding protein) and SIRT1. E2F1 was immunoprecipitated and probed with anti-Ac-K117 antibody to determine E2F1 acetylation levels. The membrane was re-probed for E2F1 and SIRT1 expression levels.

Furthermore, the ability of EX-527 to stimulate cell proliferation in wild type MEFs was not observed in *E2F1-/-* MEFs (Figure 27). These results, together with our recent finding of SIRT1-E2F1 interaction, suggest that the mechanism of cell cycle regulation by SIRT1 is in part through inhibition of E2F1.
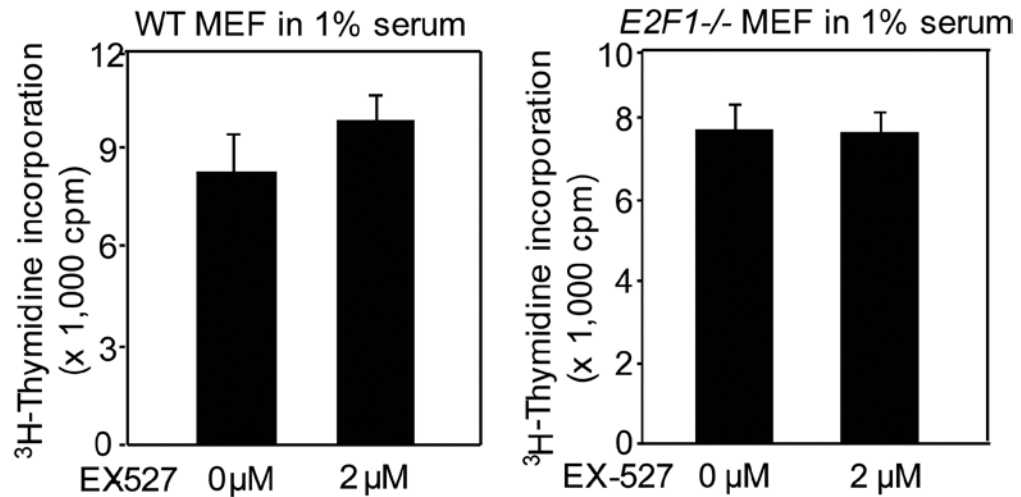
Figure 27: SIRT1 Inhibits Cell Proliferation via E2F1. Wild type and *E2F1-/-* MEFs were cultured for 18h in 1% serum and 2μM EX-527. DNA replication was measured by [3H] thymidine incorporation assay. *Error bars* represent mean $\pm$ S.D. (n=8). For wild type MEF cells, p=0.02 indicating significant difference in proliferation after treatment. For *E2F1-/-* MEFs, p=0.7, indicating no effect by EX-527 treatment.

Inhibition of SIRT1 Sensitizes Tumor Cells to Chemotherapeutic Agents

Many studies have implicated a role of SIRT1 in promoting cell survival after stress or chemotherapy. To test whether SIRT1 can exert protection against DNA damaging agents in the same HCT116 cells where it has anti-proliferative function, cells with SIRT1 knock down were treated with 5-FU, which is frequently used in colon cancer chemotherapy. SIRT1 knock down clearly reduced long term cell viability after drug treatment in a colony formation assay (Figure 28).
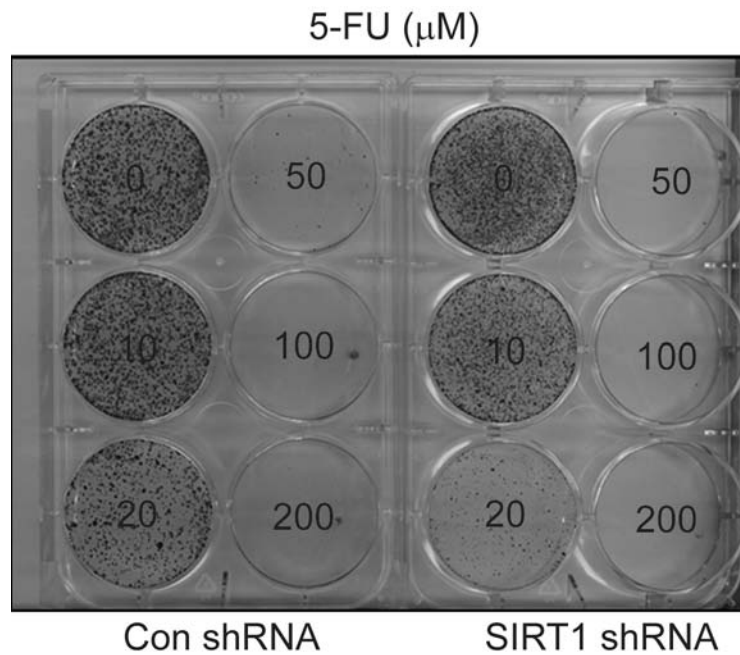
Figure 28: Inhibition of SIRT1 Increases Sensitivity to Chemotherapy. HCT116 control and *SIRT1* shRNA cell lines were treated with different concentrations of 5-FU for 24h. Cells were incubated in drug-free medium for 15 days at the end of which colonies were stained with Crystal Violet.

HCT116 cells were treated with the SIRT1 inhibitor EX-527 (2µM) and subjected to different concentrations of 5-FU. It was observed that similar to SIRT1 knock down, treatment with the SIRT1 inhibitor also reduced long term viability after exposure to 5-FU (Figure 29).
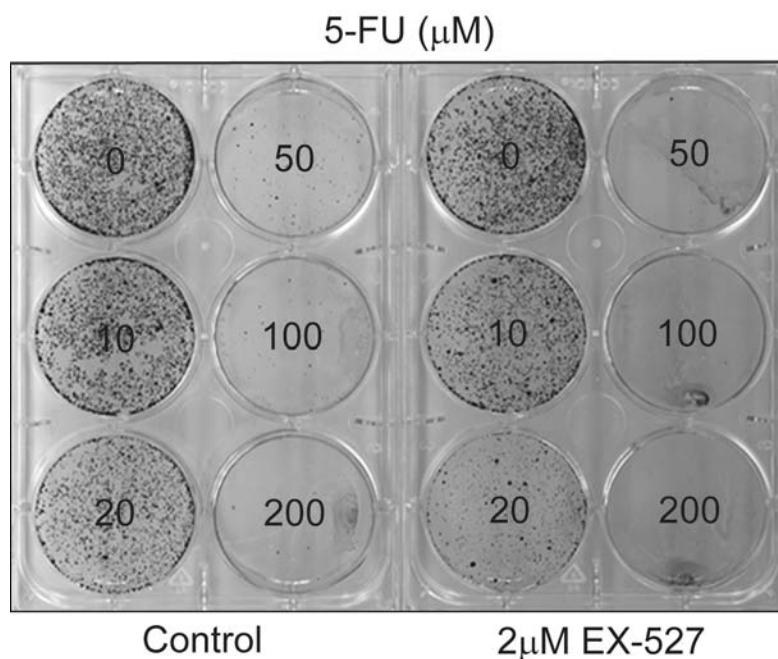
Figure 29: Inhibition of SIRT1 by EX-527 Increases Sensitivity to Chemotherapy.
HCT116 cells were treated with 2µM EX-527 and different concentrations of 5-FU for 24h.
Cells were incubated in drug-free medium for 15 days at the end of which colonies were
stained with Crystal Violet.

Besides Colony formation assays, sensitivity to chemotherapy was also measured in short

term TUNEL assays. It was observed that EX-527 enhanced (almost 100%) the ability of low

concentrations of 5-FU to induce apoptosis (10µM, Figure 30) but had no further benefit with

higher concentrations of 5-FU (50µM, data not shown).
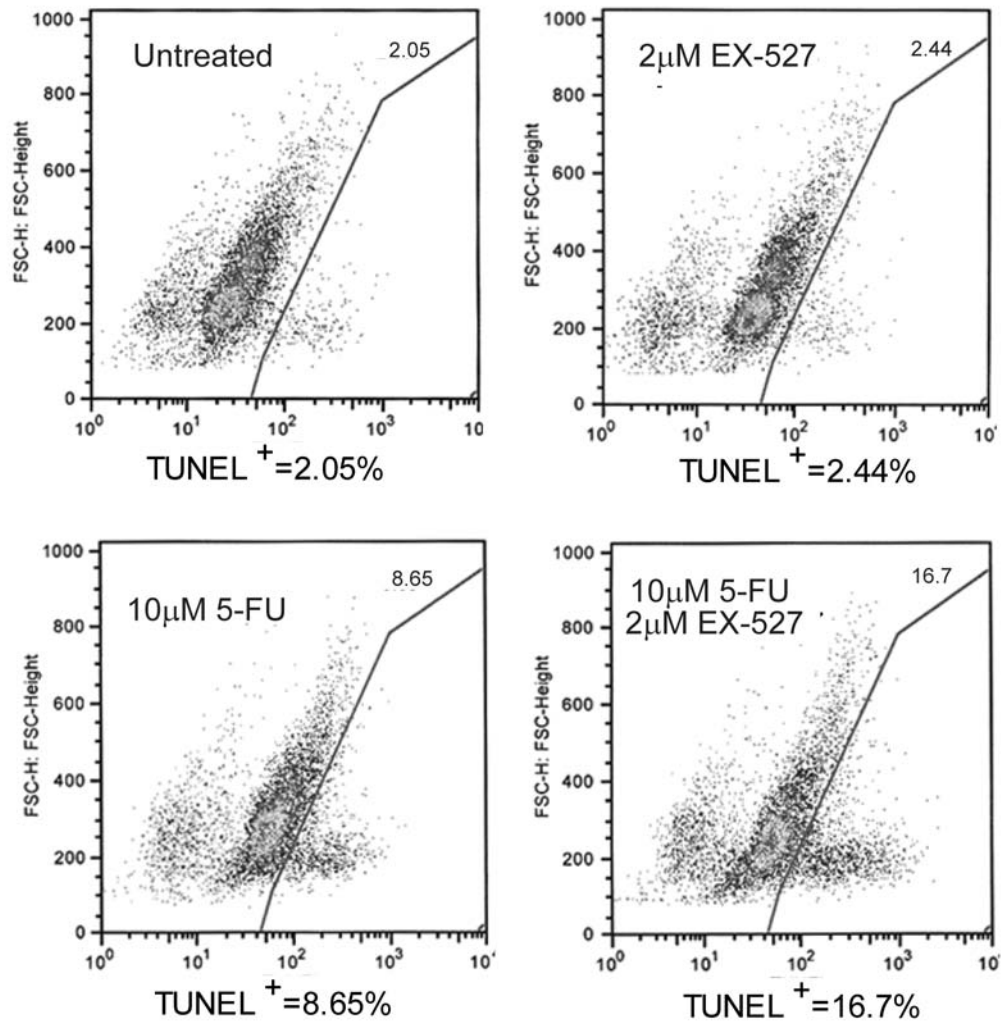
Figure 30: Inhibition of SIRT1 Promotes Cell Death in Response to Chemotherapy.
HCT116 cells were treated with 2µM EX-527 and 10µM 5-FU for 18h and analyzed by TUNEL staining and fluorescence-activated cell sorting for the presence of apoptotic cells.

EX-527 treatment also significantly enhanced the level of apoptosis in HCT116 cells induced by another colon cancer drug Camptothecin (CPT) as observed by the sub-2N population in a Fluorescence Activated Cell Sorting analysis (Figure 31).
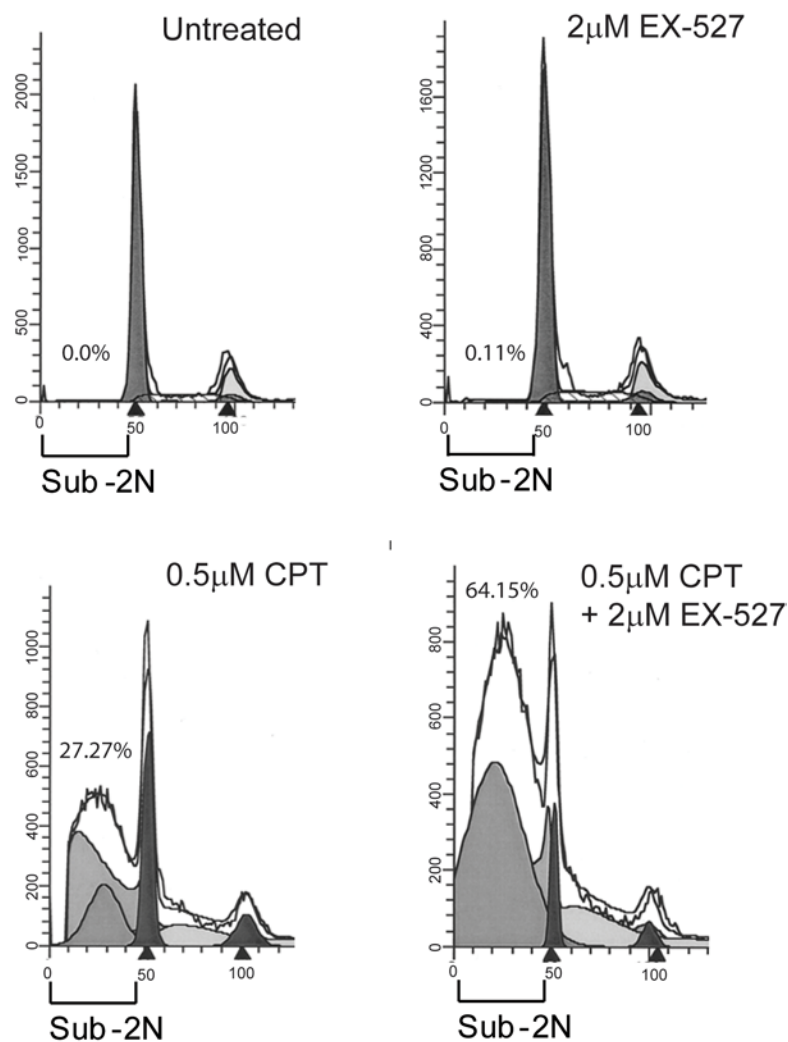
Figure 31: Inhibition of SIRT1 Increases Apoptotic Response to DNA Damage. HCT116 cells were treated with EX-527 and Camptothecin (CPT) at indicated amounts for 18h and analyzed by FACS for the presence of apoptotic sub-2N population. The raw data is represented by the curves that have not been filled in and the percentage of apoptosis is indicated in each case.

These results suggest that, under acute stress or upon DNA damage, SIRT1 expression provides a survival advantage to the cells. Because rapidly proliferating cells are often more sensitive to DNA damage-induced apoptosis, the growth-inhibitory activity of SIRT1 may be partly responsible for its anti-apoptotic function.

SIRT1 is Expressed at High Levels in Normal Colon and Benign Lesions

To investigate the role of SIRT1 in human cancer development, we developed and

characterized a monoclonal antibody 10E4 against the C-terminal domain of human SIRT1.

The antibody is highly specific for SIRT1, detects a single band on Western blot, and shows

no reactivity to other cellular proteins (Figure 32).
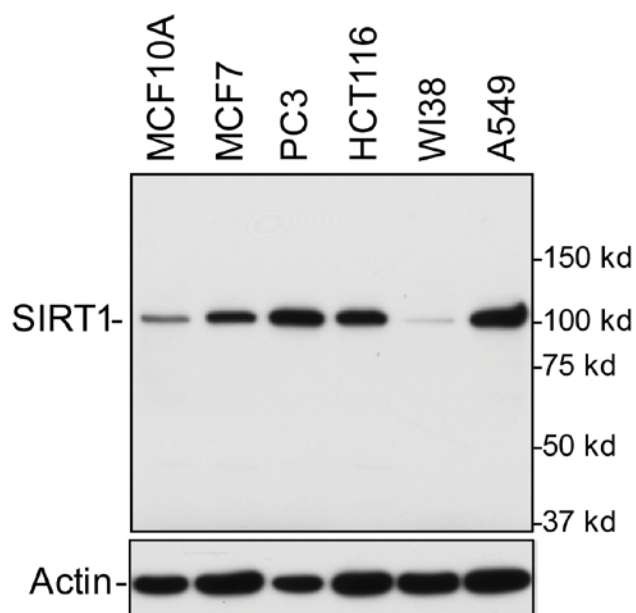
Figure 32: Characterization of SIRT1 Antibody. Detection of a single protein band by
SIRT1 monoclonal antibody,10E4, in a Western blot. Identical amounts of protein from
different cell lines were loaded in each lane.

In an immunohistochemical analysis, 10E4 detects differential nuclear staining in control cells

and SIRT1 knock down cells thereby demonstrating its ability to reveal the differences in

SIRT1 expression levels (Figure 33).
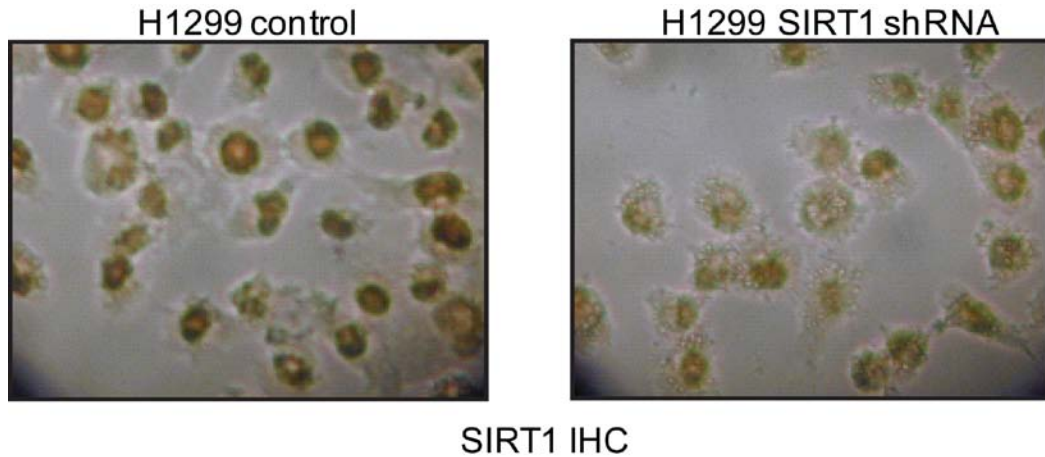
Figure 33: Characterization of SIRT1 Antibody for Immunohistochemistry.
Immunohistochemical staining (IHC) of H1299 control and SIRT1 knock down cells using
10E4 monoclonal antibody.

When normal human colon tissue samples were stained with 10E4, the results showed that

normal colon epithelial cells expressed significant levels of nuclear SIRT1 at the base of the

crypt where maximum cell proliferation occurs (*black arrows*), and the expression level

gradually decreases as cells migrate towards the lumen (*white arrows*) (Figure 34). Therefore,

SIRT1 level in normal colon epithelium correlates with active cell proliferation.

Normal mucosa (human)
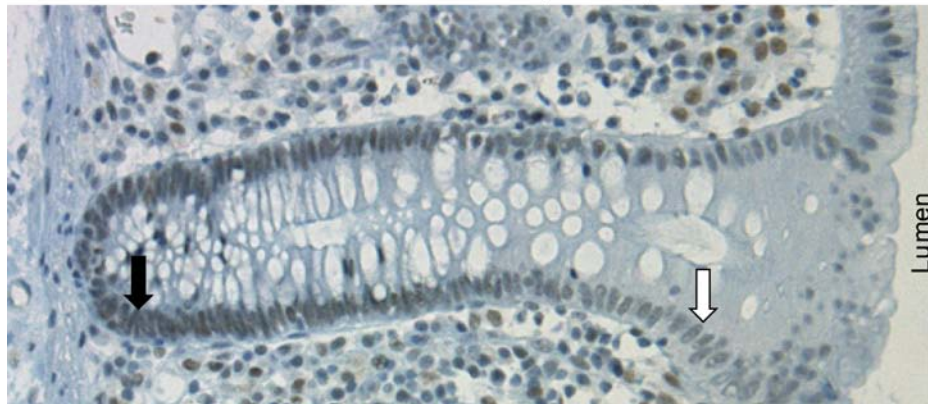
Figure 34: SIRT1 is Expressed at High Levels in Proliferating Colon Epithelium. Normal human colon mucosa was stained for SIRT1 with 10E4 monoclonal antibody. The *black arrow* indicates the base of the crypt where most cell proliferation occurs; the *white arrow* indicates cells that have migrated towards the lumen.

When benign adenomas (polyps) were stained with 10E4 and examined, nuclear SIRT1 was detected at high levels in all cells with adenomatous morphology (Figure 35, *black arrows*), but the expression was not high in the lumens of the adjacent normal mucosa (*white arrows*). This pattern was observed in all 26 adenomas that were examined, suggesting that SIRT1 is overexpressed in 100% of adenomas.
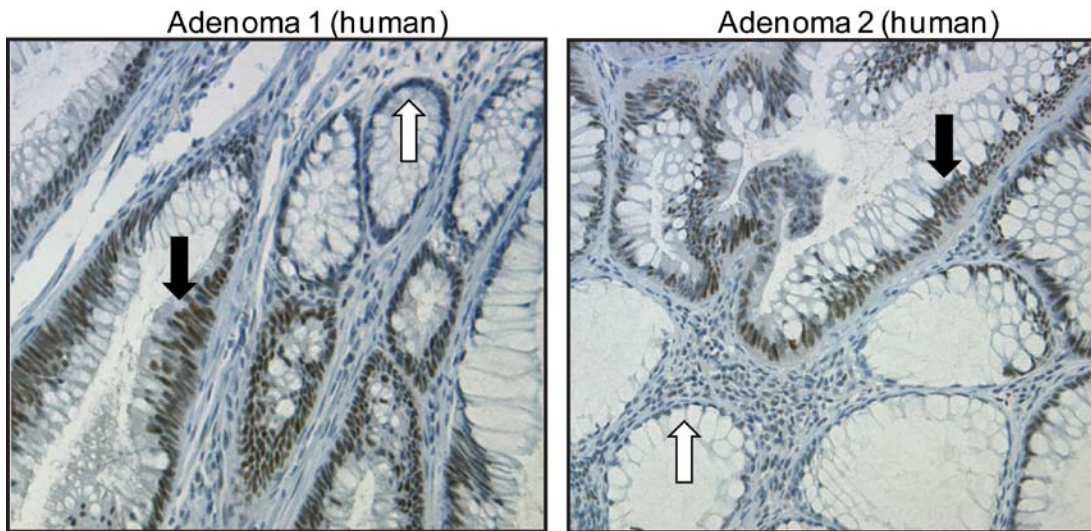
Figure 35:SIRT1 is Expressed at High Levels in Adenomatous Colon Epithelium.
Human colon adenomas were stained for SIRT1 with 10E4. The *black arrows* indicate
hyperplastic areas with dense nuclei and high levels of SIRT1 expression; *white arrows*
indicate areas of normal mucosa with low levels of SIRT1.

To test whether high SIRT1 expression is the cause or an effect of tumorigenesis, the
intestines of Apc^{Min} mice were stained for SIRT1. Apc^{Min} mice are heterozygous for codon 850
nonsense mutation in the *Apc* tumor suppressor gene. As a result of this mutation, the Apc^{Min}
mice develop intestinal adenomas by 120 days of age (Moser et al., 1990). The Apc^{Min}
adenomas develop after losing the remaining *Apc* allele and, thus, are similar to most human
colon adenomas (Luongo et al., 1994). Our results showed that SIRT1 levels were high in the
hyperplastic Apc^{Min} polyps, similar to proliferating cells near the base of the crypt in human
colon epithelium (Figure 36).
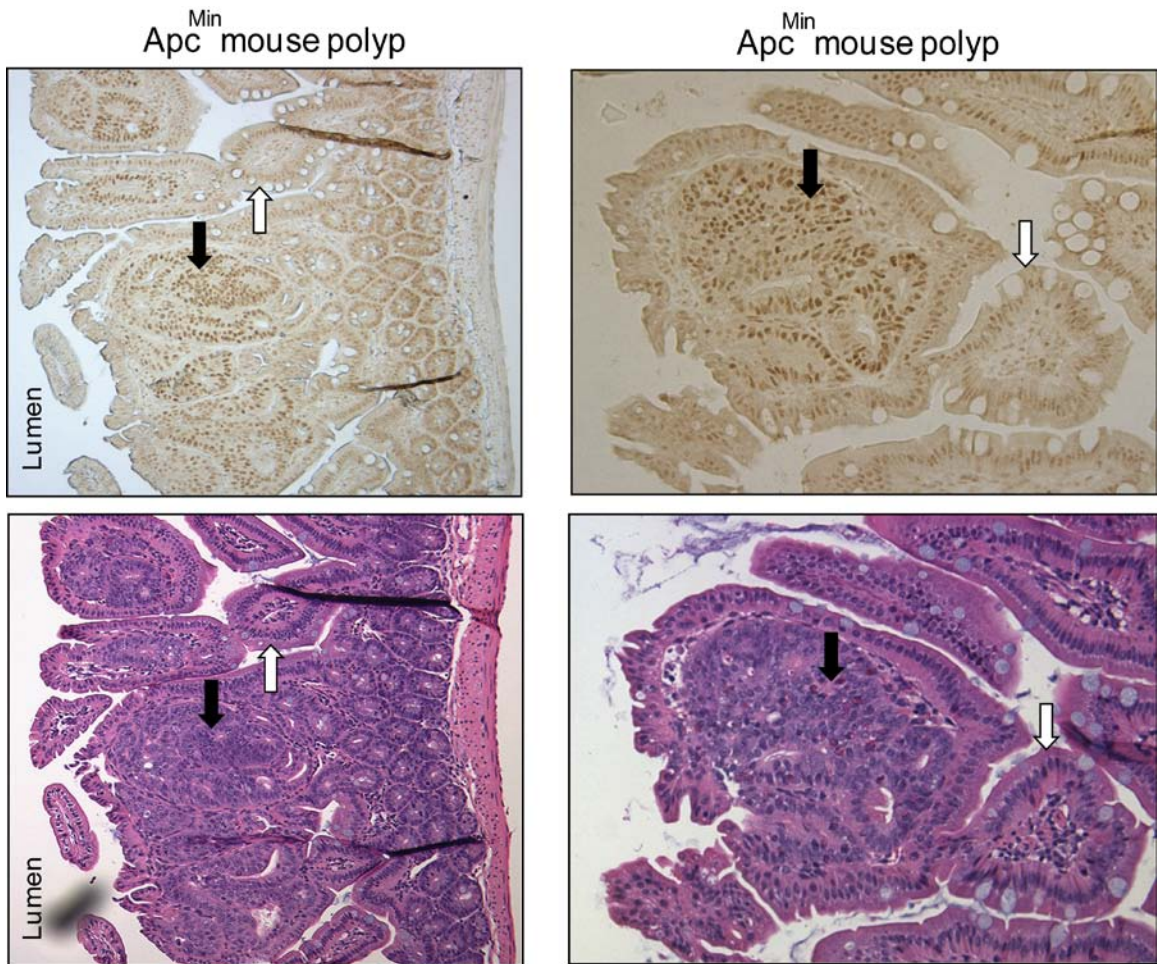
Figure 36: Sirt1 Expression in the Intestinal Lesions of Apc^{Min} Mouse. Intestinal sections of 120 day old Apc^{Min} mice were stained for Sirt1 using a polyclonal antibody against the N-terminus of mouse Sirt1 (upper panel). Corresponding tissue areas were also stained with H&E for morphological comparison (lower panel). High Sirt1 expression was maintained in the adenomatous component (black arrows) as compared to cells of the adjacent normal mucosa (white arrows).

These results suggest that SIRT1 is expressed at high levels in proliferating cells of the colon and intestinal epithelium and remains upregulated when cells undergo initial transformation. Because the Apc^{Min} polyps are initiated due to loss of the wild type *Apc* allele, increase in SIRT1 expression is most likely a secondary result of transformation and not an active cause of tumor initiation.

SIRT1 is Under- or Overexpressed in Advanced Colon Carcinomas

To determine SIRT1 expression pattern in colon tumors, the 10E4 antibody was used to stain a tissue micro array containing samples of normal colonic mucosa ($n = 11$), and of colon tumors ($n = 88$) collected at the Moffitt Cancer Center. Staining showed that SIRT1 was predominantly nuclear. When graded semi-quantitatively on a scale of 0 to 3 (0 = negative, 1= low, 2 = high, and 3 = overexpression), SIRT1 level at the base of the normal crypt and adenomas was ranked as score 2 (high). SIRT1 staining significantly more intense than adenomas was ranked as score 3 (Figure 37).
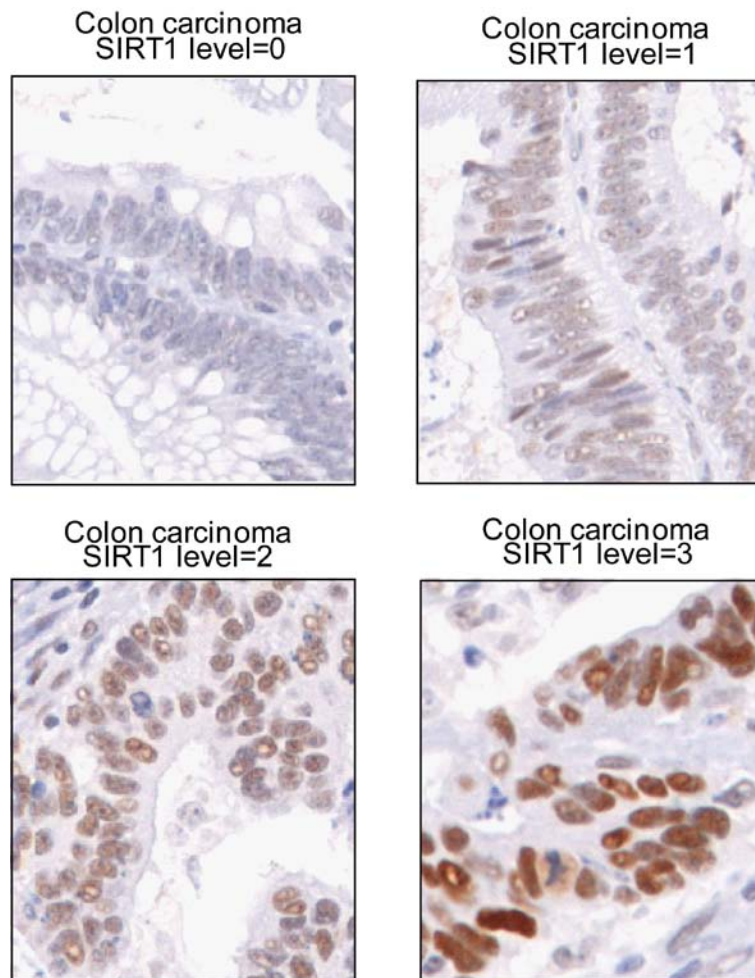
Figure 37: SIRT1 Expression is Heterogenous in Adenocarcinomas. Human colon tumor tissue array was stained for SIRT1 using 10E4. Intensity of SIRT1 stain in different tumors ranged from 0 (no stain) to 3 (overexpression).

As mentioned previously, SIRT1 expression was found to be uniformly high (score 2) in all benign adenomas. However, colonic adenocarcinomas showed a heterogeneous pattern of SIRT1 levels, ranging from score 0 to 3. Although almost 25% of stage I–III carcinomas showed an intense (score 3) nuclear staining for SIRT1, almost 30% tumors showed reduced levels of SIRT1 as compared to normal crypt and adenomas. Interestingly, only a very small subset of stage IV tumors (associated with poor 5-year survival) exhibited SIRT1 overexpression (score 3) as compared to the lower grade tumors (I–III) (Figure 38).
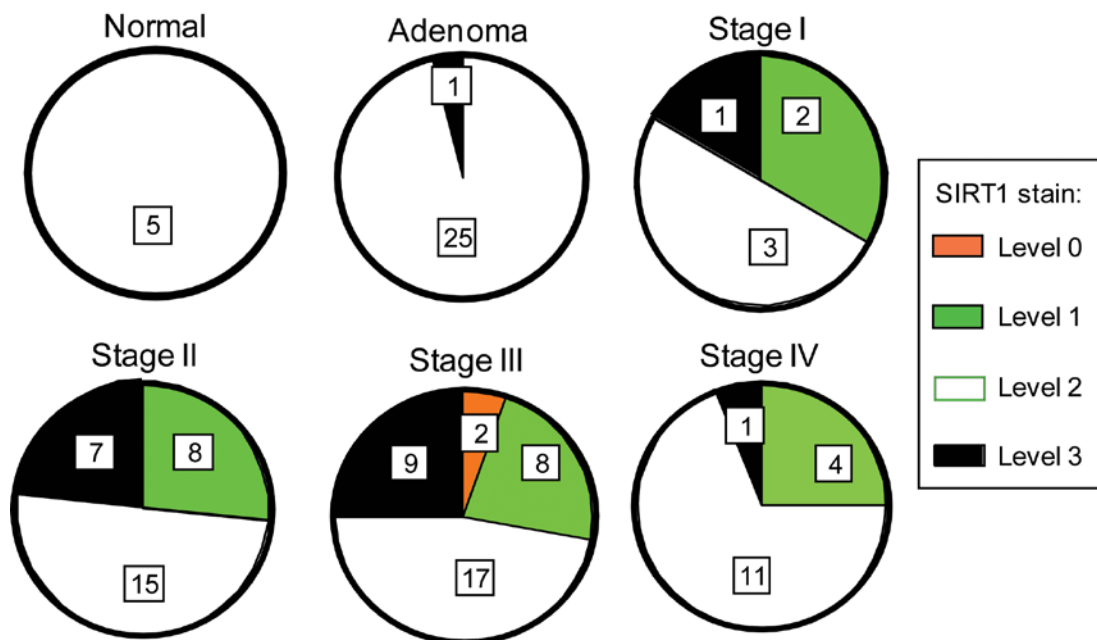
Figure 38: SIRT1 Expression is Heterogenous in Adenocarcinomas (Pie Representation). Pie representation of SIRT1 levels correlated with stage of cancer. SIRT1 level is moderate to high (level 2) in normal colonic mucosa and most adenomas. Tumors of different adenocarcinoma stages exhibit variable expression pattern of SIRT1. While some tumors overexpress SIRT1 (level 3, in black), some others downregulate SIRT1 (level 0, in orange or level 1, in green). Level 3 expression is infrequent in stage IV tumors.

Our interpretation of the heterogeneous SIRT1 expression profile is that high SIRT1 expression is an intrinsic response to cell proliferation in untransformed mucosa and pre-malignant adenomas. During further progression, SIRT1 expression is silenced in a subset of tumors to facilitate tumor growth, whereas some low grade tumors may overexpress SIRT1 to benefit from its anti-apoptotic effects. The fact that SIRT1 overexpression is rare in high grade (stage IV) tumors suggests that the growth-inhibitory activity of SIRT1 becomes a rate-limiting factor for progression to this stage.

Chapter Four

Discussion and Future Direction

The above experiments using cultured cell lines and xenograft models demonstrate that SIRT1 has properties of a growth suppressor. Knock down of SIRT1 increases the rate of tumor growth by enhancing cell proliferation, whereas overexpression of SIRT1 reduces tumor initiation and growth in nude mice. Furthermore, pharmacological inhibition of SIRT1 increases the rate of cell proliferation in culture. Interestingly, inhibition of SIRT1 also sensitizes cells to chemotherapeutic drugs. Together, these results suggest that SIRT1 functions as a context-dependent tumor suppressor where inhibition of SIRT1 promotes tumor initiation and growth but can also promote cell death upon treatment with chemotherapeutic agents presumably due to the increased sensitivity of these rapidly dividing cells to DNA damaging agents.

SIRT1 has a well established anti-apoptotic function which has led to the presumption that it acts as an oncogene. However, a recent study showed that transgenic mice overexpressing Sirt1 reduced the development of neoplasia in the intestine caused by Apc^{Min} mutation suggesting a tumor suppressive role of Sirt1 (Firestein et al., 2008). Another study demonstrated that $Sirt1+/-$ mice showed increased tumor incidence when crossed to a $p53+/-$ background (Wang et al., 2008a). These genetic models strongly suggest that SIRT1 has properties of a tumor suppressor and our results lend further support to this function of SIRT1. It is noteworthy that our results regarding the anti-proliferative and anti-tumorigenic properties of SIRT1 contradict many published studies that show an anti-apoptotic function of SIRT1. According to these published studies, tumor cells undergo apoptosis or growth arrest after

transient knock down of SIRT1 or treatment with SIRT1 inhibitors such as sirtinol, splitomycin, and cambinol (Ford et al., 2005; Heltweg et al., 2006; Ota et al., 2006). According to our results, inhibition of SIRT1 promotes cell proliferation thereby sensitizing these cells to DNA damage-induced apoptosis. A possible explanation for the discrepancies between the studies might be that, in the studies published by other research groups tumor cells treated with *SIRT1* shRNA may be sensitized to apoptosis due to additional transfection associated stress. Furthermore, the off-target toxicity of the first-generation small molecule SIRT1 inhibitors could be responsible for the cell death or growth arrest responses that they observed. In fact, recent development of the nanomolar SIRT1 inhibitor EX-527 demonstrated that specific inhibition of SIRT1 alone does not cause apoptosis in tumor cell lines (Solomon et al., 2006).

Several studies have suggested that *SIRT1* may act as an oncogene based on the correlation of higher than normal expression levels of SIRT1 in certain tumors compared to normal tissue (Hida et al., 2007; Huffman et al., 2007; Stunkel et al., 2007). Our analysis of the SIRT1 expression profile in colon cancer suggests that SIRT1 levels are variable in different stages of tumors. Such a staining pattern can be interpreted as SIRT1 having both oncogenic and tumor-suppressive properties which is consistent with the pleiotropic effects of SIRT1, *i.e.* anti-apoptotic and growth suppressive depending on cellular context. Indeed, our results may suggest that a subset of tumors downregulate SIRT1 to obtain a proliferation advantage, whereas some could increase SIRT1 expression to benefit from its anti-apoptotic function. However, interpretation of the tumor staining results is subjective and should not be taken as definitive evidence. Staining the tumor microarray for levels of ki-67 (a proliferation marker) and active caspase (apoptotic marker) would allow us to correlate SIRT1 expression levels with these cellular responses. However, it is important to consider that the level of SIRT1 may not entirely represent the enzymatic function of this protein. As mentioned in the Introduction section of this dissertation, DBC1 has been found to be an important inhibitor of SIRT1

deacetylase activity (Kim et al., 2008; Zhao et al., 2008). This implies that overexpression of DBC1 may inhibit SIRT1 activity regardless of SIRT1 expression level. Considering this, examination of the levels of DBC1 in tumor microarrays would enable us to determine the functionality of SIRT1 in tumors of different stages based on this aspect of its regulation. Although the mechanism by which SIRT1 inhibits cell proliferation remains to be further investigated, previous studies from our lab and those presented in this report suggest that inhibition of E2F1 is partly responsible for this observed effect. SIRT1 interacts with E2F1, inhibits E2F1 acetylation, and is recruited by E2F1 to target promoters (Wang et al., 2006). When expressed at high levels, SIRT1 is a potent inducer of G1 arrest. Our results show that inhibition of SIRT1 results in RB hyper-phosphorylation. This may be a result of E2F1 activation, which can induce cyclinD/cdk4 activity and promote RB phosphorylation.

Our results show that inhibition of SIRT1 promotes cell proliferation but also sensitizes the cells to apoptosis in response to DNA damaging agents presumably because cells that proliferate rapidly are more sensitive to DNA damage. In such cases, expression of SIRT1 imparts a survival advantage to the cells. Therefore, it is plausible that both activators and inhibitors of SIRT1 could have therapeutic potential as anti-tumor agents depending on the stage of cancer when treatment is adminstered. A simple scenario is that SIRT1 activators may impart cancer preventative effects by enhancing the growth-inhibitory effect of SIRT1 in benign tumors. Its effect on advanced stage tumors may be heterogeneous, depending on whether a tumor has evolved to rely on SIRT1 for survival. However, when tumors are being treated with chemotherapy, SIRT1 inhibitors may be useful for enhancing apoptotic response. Examination of a large patient cohort is needed to determine the association between SIRT1 expression and clinical parameters such as survival and treatment response, which would be critical for the application of SIRT1-targeted drugs.

To further our investigation on the function of SIRT1, we used the Apc^{Min} mouse genetic model for colon cancer. As described in the Materials and Methods section, Apc^{Min} mice were

bred to *Sirt1+/-* mice. The resulting progenies were genetically screened to obtain

Apc^{Min};*Sirt1+/+* and *Apc^{Min}*;*Sirt1+/-* mice in order to study the effect of *Sirt1* gene dosage on

polyp formation. 100% of these mice developed polyps by 6 months of age. As demonstrated

in Figure 39, no significant difference was observed in the number of polyps formed between

the two genotypes.
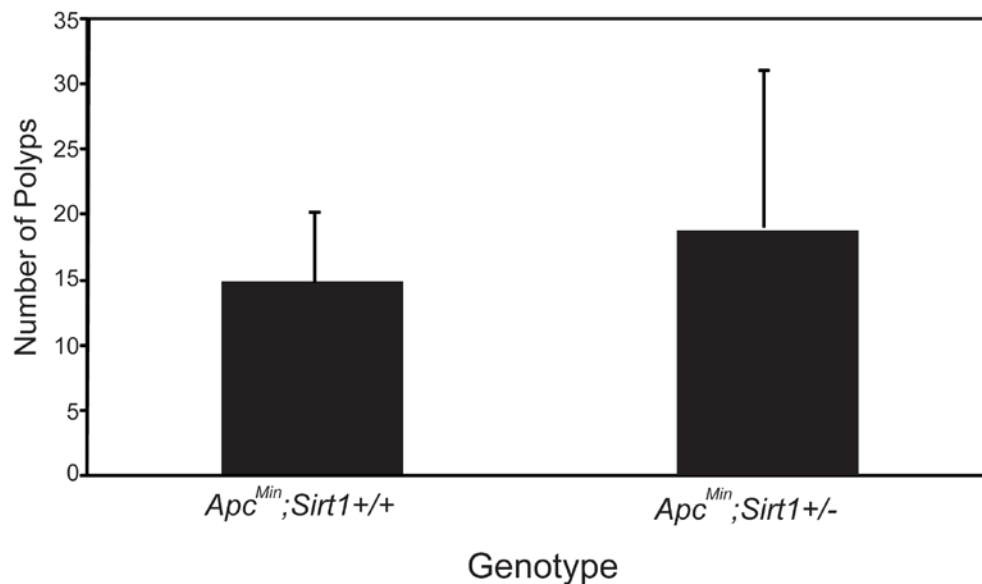
Figure 39: Effect of Sirt1 Heterozygous Genotype on Polyp Formation. Apc^{Min} mice were
bred to *Sirt1+/-* mice and the number of polyps were compared between *Apc^{Min};Sirt1+/+* and
Apc^{Min};Sirt1+/- genotypes. *Error bars* represent mean $\pm$S.D (n=12) and p=0.323 as calculated
from a two-tailed student's *t*-test.

Our hypothesis is that the heterozygous status of *Sirt1* gene may not be sufficient to show an

effect on polyp formation in this colon cancer mouse strain. Alternatively, loss of one

functional allele of *Sirt1* in the germline may have triggered additional cellular alterations such

that the effect of low Sirt1 dosage may have been compensated for and no effect was visible

on polyp formation in the intestines.

Interestingly, a recent publication showed that introduction of *Apc^Min* gene in a different *Sirt1* knockout mouse strain did not affect the number of polyps formed when compared to *Apc^Min* gene introduced in a *Sirt1* wild type background (Boily et al., 2009). However, they did notice an elevated expression of Sirt1 in the polyps of the Apc^Min mice as compared to the adjacent normal villi. In contrast, overexpression of Sirt1 has been shown to inhibit intestinal polyp formation in Apc^Min mice by downregulating β-catenin (Firestein et al., 2008). While Boily et al,. were unable to explain the reasons for this discrepancy, it could simply be a result of the different genetic backgrounds of the mouse strains that were used to perform these experiments. These observations further add to the complex nature and function of SIRT1 in tumorigenesis.

In addition to the colon cancer model, we also investigated the effect of *SIRT1* gene dosage on lung tumorigenesis using a Kras mouse lung cancer model. The K-rasLA2 mouse was obtained from the Mouse Repository (Mouse Models of Human Cancers Consortium). This mouse strain contains a latent *Kras* allele that can be activated by spontaneous mitotic recombination resulting in an active *KrasG12D* mutant allele (Johnson et al., 2001). As a result, mice heterozygous for this allele develop tumors and their mean survival age is around 200 days. Tumors mainly develop in the lungs of these mice thereby making them a suitable lung cancer model. Our studies with this model were mainly focused on two points: to determine the effect of SIRT1 on lung tumor nodule formation and to determine the effect of SIRT1 on survival of K-rasLA2 mice. K-rasLA2 mice were bred with *Sirt1+/-* mice and the *KrasG12D;Sirt1+/+* and *KrasG12D;Sirt1+/-* genotypes were selected. These mice were sacrificed at 20 weeks of age and the tumor nodules in the lung were counted. As shown in figure 40, there was no significant difference in the number of lung tumor nodules formed between the two genotypes.
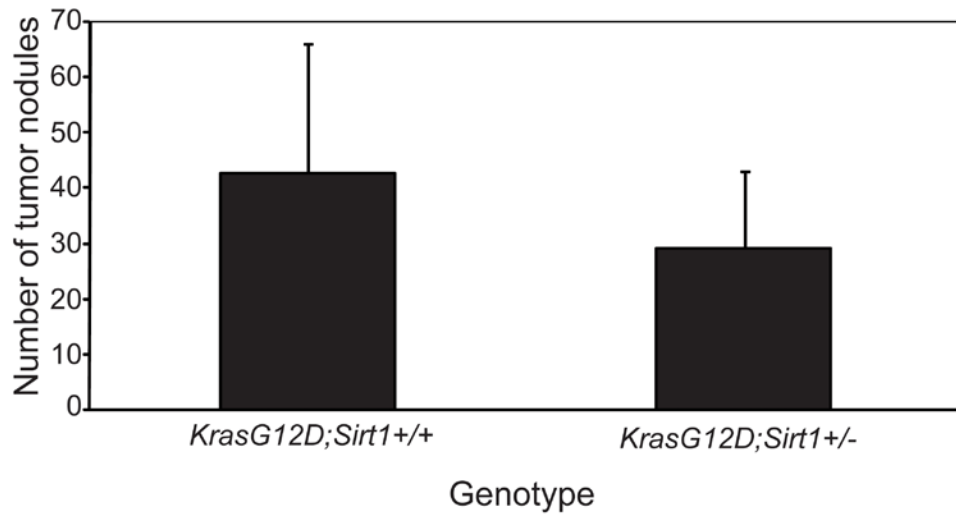
Figure 40: Effect of Sirt1 Heterozygous Genotype on Lung Tumor Nodules Formation.
K-rasLA2 mice were bred to *Sirt1+/-* mice and the indicated genotypes were sacrificed at 20
weeks of age. Number of tumor nodules were compared between *KrasG12D;Sirt1+/+* and
KrasG12D;Sirt1+/- genotypes. *Error bars* represent mean $\pm$S.D (n=12) and p=0.104 as
calculated from a two-tailed student's *t*-test.

The lungs from the above set of mice were harvested, fixed and sectioned followed by

staining for Sirt1 levels. As expected, the tumor nodules from *KrasG12D;Sirt1+/-* mouse lungs

had reduced nuclear Sirt1 staining as compared to the *KrasG12D;Sirt1+/+* lungs (Figure 41).
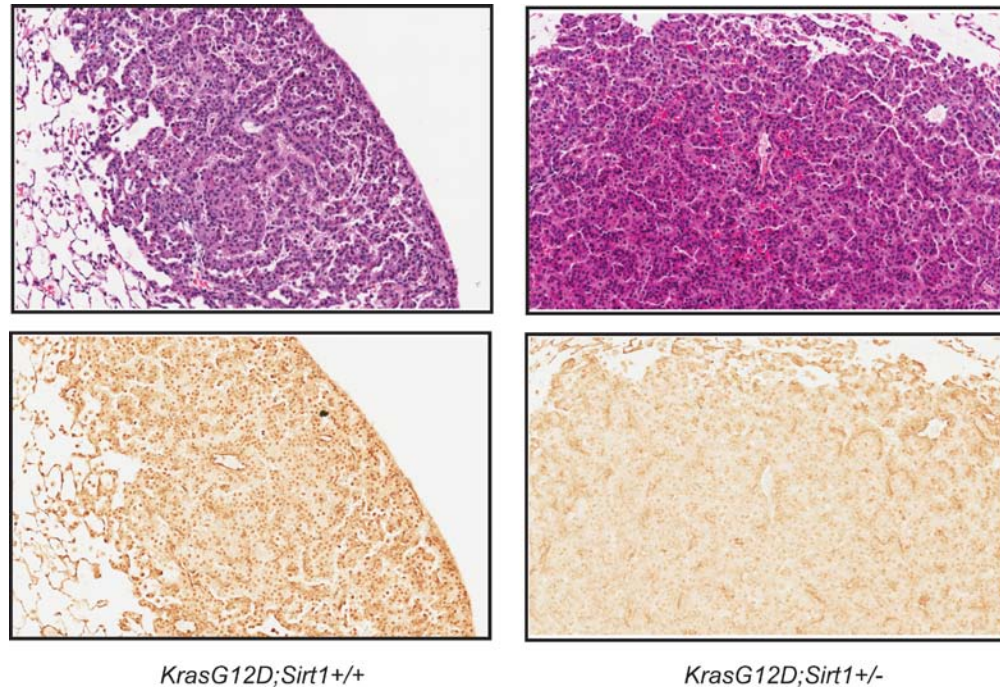
Figure 41: Lung Tumor Nodules Stained for Sirt1. Lungs from *KrasG12D;Sirt1+/+* and *KrasG12D;Sirt1+/-* mice were harvested at 20 weeks, fixed in formalin, sectioned and H&E stained (top panel). Simultaneous sections from each block were stained for Sirt1 (bottom panel) using the anti-sir2 antibody and the rabbit ABC staining system (Santa Cruz Biotech.).

To study the effect of Sirt1 on survival of the K-rasLA2 mouse strain, we were able to maintain 36 mice of *KrasG12D;Sirt1+/+* genotype and 24 mice of the *KrasG12D;Sirt1+/-* genotype for analysis. The date of birth and death for each mouse was recorded and the Kaplan Meier curve was plotted. As determined by the curve, we observed no significant difference in the survival probability between the two genotypes (p=0.37) (Figure 42). This observation leads us to suggest that partial expression of Sirt1 from one functional allele is not sufficient to affect lung tumorigenesis or mortality in this mouse model. Also, the aggressive nature of tumor formation in this mouse strain due to the Kras mutation may not allow the subtle differences between the *Sirt1+/+* and *Sirt1+/-* genotypes to surface.
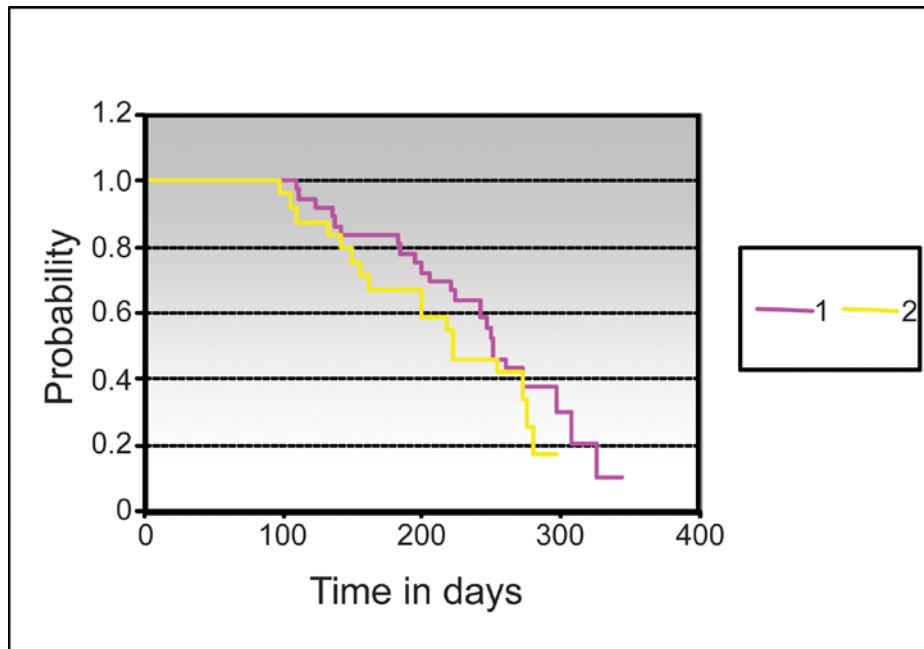
Figure 42: Comparison of Survival Probability between K-rasLA2 Mice that are Wild Type or Heterozygous for Sirt1. Pink (1) refers to *KrasG12D;Sirt1+/+* genotype and yellow (2) refers to *KrasG12D;Sirt1+/-* genotype. The chi square test with degree of freedom=1 was calculated as 0.801 and p=0.37.

The above observations suggest a complex role for SIRT1 and much research is needed to fully unravel the involvement of SIRT1 in cancer. Future research will involve the elucidation of the function of SIRT1. Lung tumor nodules from *KrasG12D;Sirt1+/+* and *KrasG12D;Sirt1+/-* mice will be used to develop cell lines. Since the Kras model was used to study only the effect of SIRT1 on tumor nodule development and overall survival, establishment of cell lines will allow us to manipulate the conditions of growth for analysis of the response of lung tumor cells to different types of stress and to determine if SIRT1 affects any of these responses. Our future plans also involve using the EX-527 SIRT1-specific inhibitor *in vivo* in the lung cancer model to effectively inhibit the deacetylase function of SIRT1. This may prove to be a better method for studying the effect of SIRT1 in lung carcinogenesis since the inhibitor will be able to achieve nearly complete inhibition of SIRT1 activity as compared to the partial inhibition

obtained from a SIRT1 heterozygous state. This may potentially provide a proof-of-concept for the use of SIRT1 inhibitor in cancer treatment. Additionally, determination of SIRT1 levels in a lung tumor microarray constructed from patient samples with more information in terms of age of patient, stage of cancer and type of therapy will be beneficial in correlating SIRT1 with patient prognosis.

References

Abdelmohsen, K., Pullmann, R., Jr., Lal, A., Kim, H. H., Galban, S., Yang, X., Blethrow, J. D., Walker, M., Shubert, J., Gillespie, D. A*., et al.* (2007). Phosphorylation of HuR by Chk2 regulates SIRT1 expression. Mol Cell *25*, 543-557.

Ahuja, N., Schwer, B., Carobbio, S., Waltregny, D., North, B. J., Castronovo, V., Maechler, P., and Verdin, E. (2007). Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. J Biol Chem *282*, 33583-33592.

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O., and Sinclair, D. A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. Nature *423*, 181-185.

Aparicio, O. M., Billington, B. L., and Gottschling, D. E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279-1287.

Ashburner, B. P., Westerheide, S. D., and Baldwin, A. S., Jr. (2001). The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol Cell Biol *21*, 7065-7077.

Bae, N. S., Swanson, M. J., Vassilev, A., and Howard, B. H. (2004). Human histone deacetylase SIRT2 interacts with the homeobox transcription factor HOXA10. J Biochem *135*, 695-700.

Bailey, D., and O'Hare, P. (2004). Characterization of the localization and proteolytic activity of the SUMO-specific protease, SENP1. J Biol Chem *279*, 692-703.

Barrett, M. T., Sanchez, C. A., Prevo, L. J., Wong, D. J., Galipeau, P. C., Paulson, T. G., Rabinovitch, P. S., and Reid, B. J. (1999). Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Genet *22*, 106-109.

Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K*., et al.* (2006). Resveratrol improves health and survival of mice on a high-calorie diet. Nature *444*, 337-342.

Bedalov, A., Gatbonton, T., Irvine, W. P., Gottschling, D. E., and Simon, J. A. (2001). Identification of a small molecule inhibitor of Sir2p. Proc Natl Acad Sci U S A *98*, 15113-15118.

Bellizzi, D., Rose, G., Cavalcante, P., Covello, G., Dato, S., De Rango, F., Greco, V., Maggiolini, M., Feraco, E., Mari, V*., et al.* (2005). A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. Genomics *85*, 258-263.

Bertos, N. R., Gilquin, B., Chan, G. K., Yen, T. J., Khochbin, S., and Yang, X. J. (2004). Role of the tetradecapeptide repeat domain of human histone deacetylase 6 in cytoplasmic retention. J Biol Chem *279*, 48246-48254.

Blander, G., Olejnik, J., Krzymanska-Olejnik, E., McDonagh, T., Haigis, M., Yaffe, M. B., and Guarente, L. (2005). SIRT1 shows no substrate specificity in vitro. J Biol Chem *280*, 9780-9785.

Boffa, L. C., Vidali, G., Mann, R. S., and Allfrey, V. G. (1978). Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. J Biol Chem *253*, 3364-3366.

Boily, G., He, X. H., Pearce, B., Jardine, K., and McBurney, M. W. (2009). SirT1-null mice develop tumors at normal rates but are poorly protected by resveratrol. Oncogene *28*, 2882-2893.

Bordone, L., Motta, M. C., Picard, F., Robinson, A., Jhala, U. S., Apfeld, J., McDonagh, T., Lemieux, M., McBurney, M., Szilvasi, A.*, et al.* (2006). Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. PLoS Biol *4*, e31.

Borra, M. T., Smith, B. C., and Denu, J. M. (2005). Mechanism of human SIRT1 activation by resveratrol. J Biol Chem *280*, 17187-17195.

Bos, J. L. (1989). ras oncogenes in human cancer: a review. Cancer Res *49*, 4682-4689.

Bouras, T., Fu, M., Sauve, A. A., Wang, F., Quong, A. A., Perkins, N. D., Hay, R. T., Gu, W., and Pestell, R. G. (2005). SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. J Biol Chem *280*, 10264-10276.

Boyault, C., Gilquin, B., Zhang, Y., Rybin, V., Garman, E., Meyer-Klaucke, W., Matthias, P., Muller, C. W., and Khochbin, S. (2006). HDAC6-p97/VCP controlled polyubiquitin chain turnover. EMBO J *25*, 3357-3366.

Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D., and Broach, J. R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev *7*, 592-604.

Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y.*, et al.* (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Buggy, J. J., Sideris, M. L., Mak, P., Lorimer, D. D., McIntosh, B., and Clark, J. M. (2000). Cloning and characterization of a novel human histone deacetylase, HDAC8. Biochem J *350 Pt 1*, 199-205.

Cahill, D. P., Kinzler, K. W., Vogelstein, B., and Lengauer, C. (1999). Genetic instability and darwinian selection in tumours. Trends Cell Biol *9*, M57-60.

Candido, E. P., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell *14*, 105-113.

Chen, D., Bruno, J., Easlon, E., Lin, S. J., Cheng, H. L., Alt, F. W., and Guarente, L. (2008). Tissue-specific regulation of SIRT1 by calorie restriction. Genes Dev *22*, 1753-1757.

Chen, W. Y., Wang, D. H., Yen, R. C., Luo, J., Gu, W., and Baylin, S. B. (2005). Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses. Cell *123*, 437-448.

Chen, Y. X., Fang, J. Y., Zhu, H. Y., Lu, R., Cheng, Z. H., and Qiu, D. K. (2004). Histone acetylation regulates p21WAF1 expression in human colon cancer cell lines. World J Gastroenterol *10*, 2643-2646.

Cheng, H. L., Mostoslavsky, R., Saito, S., Manis, J. P., Gu, Y., Patel, P., Bronson, R., Appella, E., Alt, F. W., and Chua, K. F. (2003). Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci U S A *100*, 10794-10799.

Chua, K. F., Mostoslavsky, R., Lombard, D. B., Pang, W. W., Saito, S., Franco, S., Kaushal, D., Cheng, H. L., Fischer, M. R., Stokes, N.*, et al.* (2005). Mammalian SIRT1 limits replicative life span in response to chronic genotoxic stress. Cell Metab *2*, 67-76.

Cohen, H. Y., Lavu, S., Bitterman, K. J., Hekking, B., Imahiyerobo, T. A., Miller, C., Frye, R., Ploegh, H., Kessler, B. M., and Sinclair, D. A. (2004a). Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. Mol Cell *13*, 627-638.

Cohen, H. Y., Miller, C., Bitterman, K. J., Wall, N. R., Hekking, B., Kessler, B., Howitz, K. T., Gorospe, M., de Cabo, R., and Sinclair, D. A. (2004b). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science *305*, 390-392.

Comings, D. E. (1973). A general theory of carcinogenesis. Proc Natl Acad Sci U S A *70*, 3324-3328.

Conti, J. A., Kemeny, N. E., Saltz, L. B., Huang, Y., Tong, W. P., Chou, T. C., Sun, M., Pulliam, S., and Gonzalez, C. (1996). Irinotecan is an active agent in untreated patients with metastatic colorectal cancer. J Clin Oncol *14*, 709-715.

Cooper, G. M. (1982). Cellular transforming genes. Science *217*, 801-806.

Crosby, M. E., and Almasan, A. (2004). Opposing roles of E2Fs in cell proliferation and death. Cancer Biol Ther *3*, 1208-1211.

Dai, J. M., Wang, Z. Y., Sun, D. C., Lin, R. X., and Wang, S. Q. (2007). SIRT1 interacts with p73 and suppresses p73-dependent transcriptional activity. J Cell Physiol *210*, 161-166.

David, G., Neptune, M. A., and DePinho, R. A. (2002). SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. J Biol Chem *277*, 23658-23663.

de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S., and van Kuilenburg, A. B. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J *370*, 737-749.

Denslow, S. A., and Wade, P. A. (2007). The human Mi-2/NuRD complex and gene regulation. Oncogene *26*, 5433-5438.

Dokmanovic, M., Clarke, C., and Marks, P. A. (2007). Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res *5*, 981-989.

Douillard, J. Y., Cunningham, D., Roth, A. D., Navarro, M., James, R. D., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M.*, et al.* (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet *355*, 1041-1047.

Dryden, S. C., Nahhas, F. A., Nowak, J. E., Goustin, A. S., and Tainsky, M. A. (2003). Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol Cell Biol *23*, 3173-3185.

Espinosa, J. M., and Emerson, B. M. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell *8*, 57-69.

Fattaey, A., Helin, K., and Harlow, E. (1993). Transcriptional inhibition by the retinoblastoma protein. Philos Trans R Soc Lond B Biol Sci *340*, 333-336.

Feinberg, A. P., Cui, H., and Ohlsson, R. (2002). DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. Semin Cancer Biol *12*, 389-398.

Feinberg, A. P., and Tycko, B. (2004). The history of cancer epigenetics. Nat Rev Cancer *4*, 143-153.

Firestein, R., Blander, G., Michan, S., Oberdoerffer, P., Ogino, S., Campbell, J., Bhimavarapu, A., Luikenhuis, S., de Cabo, R., Fuchs, C.*, et al.* (2008). The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth. PLoS One *3*, e2020.

Fischer, D. D., Cai, R., Bhatia, U., Asselbergs, F. A., Song, C., Terry, R., Trogani, N., Widmer, R., Atadja, P., and Cohen, D. (2002). Isolation and characterization of a novel class II histone deacetylase, HDAC10. J Biol Chem *277*, 6656-6666.

Fischle, W., Dequiedt, F., Fillion, M., Hendzel, M. J., Voelter, W., and Verdin, E. (2001). Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J Biol Chem *276*, 35826-35835.

Ford, E., Voit, R., Liszt, G., Magin, C., Grummt, I., and Guarente, L. (2006). Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. Genes Dev *20*, 1075-1080.

Ford, J., Jiang, M., and Milner, J. (2005). Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival. Cancer Res *65*, 10457-10463.

Fritze, C. E., Verschueren, K., Strich, R., and Easton Esposito, R. (1997). Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J *16*, 6495-6509.

Frye, R. A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem Biophys Res Commun *260*, 273-279.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem Biophys Res Commun *273*, 793-798.

Fu, M., Liu, M., Sauve, A. A., Jiao, X., Zhang, X., Wu, X., Powell, M. J., Yang, T., Gu, W., Avantaggiati, M. L.*, et al.* (2006). Hormonal control of androgen receptor function through SIRT1. Mol Cell Biol *26*, 8122-8135.

Gao, J., Siddoway, B., Huang, Q., and Xia, H. (2009). Inactivation of CREB mediated gene transcription by HDAC8 bound protein phosphatase. Biochem Biophys Res Commun *379*, 1-5.

Gao, L., Cueto, M. A., Asselbergs, F., and Atadja, P. (2002). Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J Biol Chem *277*, 25748-25755.

Gerhart-Hines, Z., Rodgers, J. T., Bare, O., Lerin, C., Kim, S. H., Mostoslavsky, R., Alt, F. W., Wu, Z., and Puigserver, P. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. EMBO J *26*, 1913-1923.

Giono, L. E., and Manfredi, J. J. (2006). The p53 tumor suppressor participates in multiple cell cycle checkpoints. J Cell Physiol *209*, 13-20.

Glozak, M. A., and Seto, E. (2007). Histone deacetylases and cancer. Oncogene *26*, 5420-5432.

Glozak, M. A., and Seto, E. (2009). Acetylation/deacetylation modulates the stability of DNA replication licensing factor Cdt1. J Biol Chem *284*, 11446-11453.

Gottlieb, S., and Esposito, R. E. (1989). A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. Cell *56*, 771-776.

Gregoretti, I. V., Lee, Y. M., and Goodson, H. V. (2004). Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol *338*, 17-31.

Grozinger, C. M., Chao, E. D., Blackwell, H. E., Moazed, D., and Schreiber, S. L. (2001). Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J Biol Chem *276*, 38837-38843.

Grozinger, C. M., Hassig, C. A., and Schreiber, S. L. (1999). Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A *96*, 4868-4873.

Grozinger, C. M., and Schreiber, S. L. (2000). Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc Natl Acad Sci U S A *97*, 7835-7840.

Grozinger, C. M., and Schreiber, S. L. (2002). Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol *9*, 3-16.

Grzenda, A., Lomberk, G., Zhang, J. S., and Urrutia, R. (2009). Sin3: master scaffold and transcriptional corepressor. Biochim Biophys Acta *1789*, 443-450.

Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

Guardiola, A. R., and Yao, T. P. (2002). Molecular cloning and characterization of a novel histone deacetylase HDAC10. J Biol Chem *277*, 3350-3356.

Guarente, L. (2000). Sir2 links chromatin silencing, metabolism, and aging. Genes Dev *14*, 1021-1026.

Haber, M., and Stewart, B. W. (1985). Oncogenes. A possible role for cancer genes in human malignant disease. Med J Aust *142*, 402-406.

Haberland, M., Arnold, M. A., McAnally, J., Phan, D., Kim, Y., and Olson, E. N. (2007). Regulation of HDAC9 gene expression by MEF2 establishes a negative-feedback loop in the transcriptional circuitry of muscle differentiation. Mol Cell Biol *27*, 518-525.

Haigis, M. C., Mostoslavsky, R., Haigis, K. M., Fahie, K., Christodoulou, D. C., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Karow, M., Blander, G.*, et al.* (2006). SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. Cell *126*, 941-954.

Halkidou, K., Gaughan, L., Cook, S., Leung, H. Y., Neal, D. E., and Robson, C. N. (2004). Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate *59*, 177-189.

Hall, S. S. (2003). Longevity research. In vino vitalis? Compounds activate life-extending genes. Science *301*, 1165.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harbour, J. W., and Dean, D. C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev *14*, 2393-2409.

Helin, K., Lees, J. A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A. (1992). A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell *70*, 337-350.

Heltweg, B., Gatbonton, T., Schuler, A. D., Posakony, J., Li, H., Goehle, S., Kollipara, R., Depinho, R. A., Gu, Y., Simon, J. A., and Bedalov, A. (2006). Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. Cancer Res *66*, 4368-4377.

Hida, Y., Kubo, Y., Murao, K., and Arase, S. (2007). Strong expression of a longevity-related protein, SIRT1, in Bowen's disease. Arch Dermatol Res *299*, 103-106.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. L*., et al.* (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature *425*, 191-196.

Hsiang, Y. H., Hertzberg, R., Hecht, S., and Liu, L. F. (1985). Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J Biol Chem *260*, 14873-14878.
Hu, E., Chen, Z., Fredrickson, T., Zhu, Y., Kirkpatrick, R., Zhang, G. F., Johanson, K., Sung, C. M., Liu, R., and Winkler, J. (2000). Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. J Biol Chem *275*, 15254-15264.

Huang, B. H., Laban, M., Leung, C. H., Lee, L., Lee, C. K., Salto-Tellez, M., Raju, G. C., and Hooi, S. C. (2005). Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. Cell Death Differ *12*, 395-404.

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., and Yao, T. P. (2002). HDAC6 is a microtubule-associated deacetylase. Nature *417*, 455-458.

Huffman, D. M., Grizzle, W. E., Bamman, M. M., Kim, J. S., Eltoum, I. A., Elgavish, A., and Nagy, T. R. (2007). SIRT1 is significantly elevated in mouse and human prostate cancer. Cancer Res *67*, 6612-6618.

Huhtiniemi, T., Wittekindt, C., Laitinen, T., Leppanen, J., Salminen, A., Poso, A., and Lahtela-Kakkonen, M. (2006). Comparative and pharmacophore model for deacetylase SIRT1. J Comput Aided Mol Des *20*, 589-599.

Inoue, T., Hiratsuka, M., Osaki, M., and Oshimura, M. (2007). The molecular biology of mammalian SIRT proteins: SIRT2 in cell cycle regulation. Cell Cycle *6*, 1011-1018.

Jackson, M. D., Schmidt, M. T., Oppenheimer, N. J., and Denu, J. M. (2003). Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. J Biol Chem *278*, 50985-50998.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Johnson, L., Mercer, K., Greenbaum, D., Bronson, R. T., Crowley, D., Tuveson, D. A., and Jacks, T. (2001). Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature *410*, 1111-1116.

Kaeberlein, M., McDonagh, T., Heltweg, B., Hixon, J., Westman, E. A., Caldwell, S. D., Napper, A., Curtis, R., DiStefano, P. S., Fields, S*., et al.* (2005). Substrate-specific activation of sirtuins by resveratrol. J Biol Chem *280*, 17038-17045.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev *13*, 2570-2580.

Kamel, C., Abrol, M., Jardine, K., He, X., and McBurney, M. W. (2006). SirT1 fails to affect p53-mediated biological functions. Aging Cell *5*, 81-88.

Kanfi, Y., Peshti, V., Gozlan, Y. M., Rathaus, M., Gil, R., and Cohen, H. Y. (2008a). Regulation of SIRT1 protein levels by nutrient availability. FEBS Lett *582*, 2417-2423.

Kanfi, Y., Shalman, R., Peshti, V., Pilosof, S. N., Gozlan, Y. M., Pearson, K. J., Lerrer, B., Moazed, D., Marine, J. C., de Cabo, R., and Cohen, H. Y. (2008b). Regulation of SIRT6 protein levels by nutrient availability. FEBS Lett *582*, 543-548.

Kang, H., Jung, J. W., Kim, M. K., and Chung, J. H. (2009). CK2 is the regulator of SIRT1 substrate-binding affinity, deacetylase activity and cellular response to DNA-damage. PLoS One *4*, e6611.

Kao, H. Y., Lee, C. H., Komarov, A., Han, C. C., and Evans, R. M. (2002). Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. J Biol Chem *277*, 187-193.

Kawahara, T. L., Michishita, E., Adler, A. S., Damian, M., Berber, E., Lin, M., McCord, R. A., Ongaigui, K. C., Boxer, L. D., Chang, H. Y., and Chua, K. F. (2009). SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. Cell *136*, 62-74.

Kim, E. J., Kho, J. H., Kang, M. R., and Um, S. J. (2007). Active regulator of SIRT1 cooperates with SIRT1 and facilitates suppression of p53 activity. Mol Cell *28*, 277-290.

Kim, E. J., and Um, S. J. (2008). SIRT1: roles in aging and cancer. BMB Rep *41*, 751-756.

Kim, J. E., Chen, J., and Lou, Z. (2008). DBC1 is a negative regulator of SIRT1. Nature *451*, 583-586.

Kloting, N., and Bluher, M. (2005). Extended longevity and insulin signaling in adipose tissue. Exp Gerontol *40*, 878-883.

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A *68*, 820-823.

Kovacs, J. J., Murphy, P. J., Gaillard, S., Zhao, X., Wu, J. T., Nicchitta, C. V., Yoshida, M., Toft, D. O., Pratt, W. B., and Yao, T. P. (2005). HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Mol Cell *18*, 601-607.

Kruszewski, M., and Szumiel, I. (2005). Sirtuins (histone deacetylases III) in the cellular response to DNA damage--facts and hypotheses. DNA Repair (Amst) *4*, 1306-1313.

Lagger, G., O'Carroll, D., Rembold, M., Khier, H., Tischler, J., Weitzer, G., Schuettengruber, B., Hauser, C., Brunmeir, R., Jenuwein, T., and Seiser, C. (2002). Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J *21*, 2672-2681.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P*., et al.* (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell *127*, 1109-1122.

Lai, A., Lee, J. M., Yang, W. M., DeCaprio, J. A., Kaelin, W. G., Jr., Seto, E., and Branton, P. E. (1999). RBP1 recruits both histone deacetylase-dependent and -independent repression activities to retinoblastoma family proteins. Mol Cell Biol *19*, 6632-6641.

Lain, S., Hollick, J. J., Campbell, J., Staples, O. D., Higgins, M., Aoubala, M., McCarthy, A., Appleyard, V., Murray, K. E., Baker, L*., et al.* (2008). Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. Cancer Cell *13*, 454-463.

Landry, J., Slama, J. T., and Sternglanz, R. (2000). Role of NAD(+) in the deacetylase activity of the SIR2-like proteins. Biochem Biophys Res Commun *278*, 685-690.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Lane, D. P., and Crawford, L. V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261-263.

Langley, E., Pearson, M., Faretta, M., Bauer, U. M., Frye, R. A., Minucci, S., Pelicci, P. G., and Kouzarides, T. (2002). Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J *21*, 2383-2396.

Lee, H., Rezai-Zadeh, N., and Seto, E. (2004). Negative regulation of histone deacetylase 8 activity by cyclic AMP-dependent protein kinase A. Mol Cell Biol *24*, 765-773.

Lee, I. H., Cao, L., Mostoslavsky, R., Lombard, D. B., Liu, J., Bruns, N. E., Tsokos, M., Alt, F. W., and Finkel, T. (2008). A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proc Natl Acad Sci U S A *105*, 3374-3379.

Lee, J. H., Song, M. Y., Song, E. K., Kim, E. K., Moon, W. S., Han, M. K., Park, J. W., Kwon, K. B., and Park, B. H. (2009). Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway. Diabetes *58*, 344-351.

Lemercier, C., Verdel, A., Galloo, B., Curtet, S., Brocard, M. P., and Khochbin, S. (2000). mHDA1/HDAC5 histone deacetylase interacts with and represses MEF2A transcriptional activity. J Biol Chem *275*, 15594-15599.

Li, X., Song, S., Liu, Y., Ko, S. H., and Kao, H. Y. (2004). Phosphorylation of the histone deacetylase 7 modulates its stability and association with 14-3-3 proteins. J Biol Chem *279*, 34201-34208.

Li, Z., Chen, L., Kabra, N., Wang, C., Fang, J., and Chen, J. (2009). Inhibition of SUV39H1 methyltransferase activity by DBC1. J Biol Chem *284*, 10361-10366.

Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997). Binding and modulation of p53 by p300/CBP coactivators. Nature *387*, 823-827.

Lin, S. J., Defossez, P. A., and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science *289*, 2126-2128.

Lin, S. J., Kaeberlein, M., Andalis, A. A., Sturtz, L. A., Defossez, P. A., Culotta, V. C., Fink, G. R., and Guarente, L. (2002). Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature *418*, 344-348.

Liszt, G., Ford, E., Kurtev, M., and Guarente, L. (2005). Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. J Biol Chem *280*, 21313-21320.
Liu, T., Liu, P. Y., and Marshall, G. M. (2009). The critical role of the class III histone deacetylase SIRT1 in cancer. Cancer Res *69*, 1702-1705.

Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137-148.

Luo, R. X., Postigo, A. A., and Dean, D. C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463-473.

Luongo, C., Moser, A. R., Gledhill, S., and Dove, W. F. (1994). Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res *54*, 5947-5952.

Markowitz, S. D., and Bertagnolli, M. M. (2009). Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med *361*, 2449-2460.

Marks, P. A., and Breslow, R. (2007). Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol *25*, 84-90.

Matlashewski, G., Lamb, P., Pim, D., Peacock, J., Crawford, L., and Benchimol, S. (1984). Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. EMBO J *3*, 3257-3262.

Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S.*, et al.* (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J *21*, 6820-6831.

McBurney, M. W., Yang, X., Jardine, K., Hixon, M., Boekelheide, K., Webb, J. R., Lansdorp, P. M., and Lemieux, M. (2003). The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol Cell Biol *23*, 38-54.

Michan, S., and Sinclair, D. (2007). Sirtuins in mammals: insights into their biological function. Biochem J *404*, 1-13.

Michishita, E., McCord, R. A., Berber, E., Kioi, M., Padilla-Nash, H., Damian, M., Cheung, P., Kusumoto, R., Kawahara, T. L., Barrett, J. C.*, et al.* (2008). SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature *452*, 492-496.

Michishita, E., McCord, R. A., Boxer, L. D., Barber, M. F., Hong, T., Gozani, O., and Chua, K. F. (2009). Cell cycle-dependent deacetylation of telomeric histone H3 lysine K56 by human SIRT6. Cell Cycle *8*, 2664-2666.

Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C., and Horikawa, I. (2005). Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell *16*, 4623-4635.

Milburn, M. V., Tong, L., deVos, A. M., Brunger, A., Yamaizumi, Z., Nishimura, S., and Kim, S. H. (1990). Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. Science *247*, 939-945.

Milne, J. C., Lambert, P. D., Schenk, S., Carney, D. P., Smith, J. J., Gagne, D. J., Jin, L., Boss, O., Perni, R. B., Vu, C. B.*, et al.* (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature *450*, 712-716.

Miska, E. A., Karlsson, C., Langley, E., Nielsen, S. J., Pines, J., and Kouzarides, T. (1999). HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J *18*, 5099-5107.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293-299.

Moser, A. R., Pitot, H. C., and Dove, W. F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science *247*, 322-324.

Mostoslavsky, R., Chua, K. F., Lombard, D. B., Pang, W. W., Fischer, M. R., Gellon, L., Liu, P., Mostoslavsky, G., Franco, S., Murphy, M. M.*, et al.* (2006). Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell *124*, 315-329.

Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551-563.

Moynihan, K. A., Grimm, A. A., Plueger, M. M., Bernal-Mizrachi, E., Ford, E., Cras-Meneur, C., Permutt, M. A., and Imai, S. (2005). Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. Cell Metab *2*, 105-117.

Muller, S., Ledl, A., and Schmidt, D. (2004). SUMO: a regulator of gene expression and genome integrity. Oncogene *23*, 1998-2008.

Murayama, A., Ohmori, K., Fujimura, A., Minami, H., Yasuzawa-Tanaka, K., Kuroda, T., Oie, S., Daitoku, H., Okuwaki, M., Nagata, K.*, et al.* (2008). Epigenetic control of rDNA loci in response to intracellular energy status. Cell *133*, 627-639.

Nakagawa, T., Lomb, D. J., Haigis, M. C., and Guarente, L. (2009). SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. Cell *137*, 560-570.

Napper, A. D., Hixon, J., McDonagh, T., Keavey, K., Pons, J. F., Barker, J., Yau, W. T., Amouzegh, P., Flegg, A., Hamelin, E.*, et al.* (2005). Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. J Med Chem *48*, 8045-8054.

Nemoto, S., Fergusson, M. M., and Finkel, T. (2004). Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. Science *306*, 2105-2108.

Nemoto, S., Fergusson, M. M., and Finkel, T. (2005). SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. J Biol Chem *280*, 16456-16460.

Nie, Y., Erion, D. M., Yuan, Z., Dietrich, M., Shulman, G. I., Horvath, T. L., and Gao, Q. (2009). STAT3 inhibition of gluconeogenesis is downregulated by SirT1. Nat Cell Biol *11*, 492-500.

North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M., and Verdin, E. (2003). The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell *11*, 437-444.

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. Science *194*, 23-28.

Olsson, A., Manzl, C., Strasser, A., and Villunger, A. (2007). How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? Cell Death Differ *14*, 1561-1575.

Onyango, P., Celic, I., McCaffery, J. M., Boeke, J. D., and Feinberg, A. P. (2002). SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. Proc Natl Acad Sci U S A *99*, 13653-13658.

Ota, H., Tokunaga, E., Chang, K., Hikasa, M., Iijima, K., Eto, M., Kozaki, K., Akishita, M., Ouchi, Y., and Kaneki, M. (2006). Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. Oncogene *25*, 176-185.

Pacholec, M., Chrunyk, B. A., Cunningham, D., Flynn, D., Griffith, D. A., Griffor, M., Loulakis, P., Pabst, B., Qiu, X., Stockman, B.*, et al.* (2010). SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J Biol Chem.

Pajak, B., Orzechowski, A., and Gajkowska, B. (2007). Molecular basis of sodium butyrate-dependent proapoptotic activity in cancer cells. Adv Med Sci *52*, 83-88.

Pediconi, N., Guerrieri, F., Vossio, S., Bruno, T., Belloni, L., Schinzari, V., Scisciani, C., Fanciulli, M., and Levrero, M. (2009). hSirT1-dependent regulation of the PCAF-E2F1-p73 apoptotic pathway in response to DNA damage. Mol Cell Biol *29*, 1989-1998.

Perrod, S., Cockell, M. M., Laroche, T., Renauld, H., Ducrest, A. L., Bonnard, C., and Gasser, S. M. (2001). A cytosolic NAD-dependent deacetylase, Hst2p, can modulate nucleolar and telomeric silencing in yeast. EMBO J *20*, 197-209.

Pflum, M. K., Tong, J. K., Lane, W. S., and Schreiber, S. L. (2001). Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation. J Biol Chem *276*, 47733-47741.

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M. W., and Guarente, L. (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature *429*, 771-776.

Pitot, H. C., and Dragan, Y. P. (1991). Facts and theories concerning the mechanisms of carcinogenesis. FASEB J *5*, 2280-2286.

Ramsey, K. M., Mills, K. F., Satoh, A., and Imai, S. (2008). Age-associated loss of Sirt1-mediated enhancement of glucose-stimulated insulin secretion in beta cell-specific Sirt1-overexpressing (BESTO) mice. Aging Cell *7*, 78-88.

Reed, N. A., Cai, D., Blasius, T. L., Jih, G. T., Meyhofer, E., Gaertig, J., and Verhey, K. J. (2006). Microtubule acetylation promotes kinesin-1 binding and transport. Curr Biol *16*, 2166-2172.

Richon, V. M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R. A., and Marks, P. A. (1998). A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A *95*, 3003-3007.

Riesen, M., and Morgan, A. (2009). Calorie restriction reduces rDNA recombination independently of rDNA silencing. Aging Cell *8*, 624-632.

Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L., and Wolffe, A. P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet *25*, 338-342.

Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., and Puigserver, P. (2005). Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature *434*, 113-118.

Rogina, B., and Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc Natl Acad Sci U S A *101*, 15998-16003.

Roth, G. S., Ingram, D. K., and Lane, M. A. (1999). Calorie restriction in primates: will it work and how will we know? J Am Geriatr Soc *47*, 896-903.

Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet *25*, 269-277.

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev *12*, 2831-2841.

Salminen, A., and Kaarniranta, K. (2009). SIRT1: regulation of longevity via autophagy. Cell Signal *21*, 1356-1360.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., Maroun, J. A., Ackland, S. P., Locker, P. K., Pirotta, N.*, et al.* (2000). Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med *343*, 905-914.

Sambucetti, L. C., Fischer, D. D., Zabludoff, S., Kwon, P. O., Chamberlin, H., Trogani, N., Xu, H., and Cohen, D. (1999). Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. J Biol Chem *274*, 34940-34947.

Sasaki, T., Maier, B., Koclega, K. D., Chruszcz, M., Gluba, W., Stukenberg, P. T., Minor, W., and Scrable, H. (2008). Phosphorylation regulates SIRT1 function. PLoS One *3*, e4020.

Scher, M. B., Vaquero, A., and Reinberg, D. (2007). SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress. Genes Dev *21*, 920-928.

Schlicker, C., Gertz, M., Papatheodorou, P., Kachholz, B., Becker, C. F., and Steegborn, C. (2008). Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. J Mol Biol *382*, 790-801.

Sealy, L., and Chalkley, R. (1978). The effect of sodium butyrate on histone modification. Cell *14*, 115-121.

Segal, N. H., and Saltz, L. B. (2009). Evolving treatment of advanced colon cancer. Annu Rev Med *60*, 207-219.

Seigneurin-Berny, D., Verdel, A., Curtet, S., Lemercier, C., Garin, J., Rousseaux, S., and Khochbin, S. (2001). Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways. Mol Cell Biol *21*, 8035-8044.

Sengupta, N., and Seto, E. (2004). Regulation of histone deacetylase activities. J Cell Biochem *93*, 57-67.

Sherr, C. J. (2004). Principles of tumor suppression. Cell *116*, 235-246.

Shi, T., Wang, F., Stieren, E., and Tong, Q. (2005). SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem *280*, 13560-13567.

Sinclair, D. A., and Guarente, L. (1997). Extrachromosomal rDNA circles--a cause of aging in yeast. Cell *91*, 1033-1042.

Smith, J. (2002). Human Sir2 and the 'silencing' of p53 activity. Trends Cell Biol *12*, 404-406.

Solomon, J. M., Pasupuleti, R., Xu, L., McDonagh, T., Curtis, R., DiStefano, P. S., and Huber, L. J. (2006). Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. Mol Cell Biol *26*, 28-38.

Strahl, B. D., and Allis, C. D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

Stunkel, W., Peh, B. K., Tan, Y. C., Nayagam, V. M., Wang, X., Salto-Tellez, M., Ni, B., Entzeroth, M., and Wood, J. (2007). Function of the SIRT1 protein deacetylase in cancer. Biotechnol J *2*, 1360-1368.

Sun, C., Zhang, F., Ge, X., Yan, T., Chen, X., Shi, X., and Zhai, Q. (2007). SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. Cell Metab *6*, 307-319.

Takami, Y., and Nakayama, T. (2000). N-terminal region, C-terminal region, nuclear export signal, and deacetylation activity of histone deacetylase-3 are essential for the viability of the DT40 chicken B cell line. J Biol Chem *275*, 16191-16201.

Tanner, K. G., Landry, J., Sternglanz, R., and Denu, J. M. (2000). Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. Proc Natl Acad Sci U S A *97*, 14178-14182.

Tanno, M., Sakamoto, J., Miura, T., Shimamoto, K., and Horio, Y. (2007). Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. J Biol Chem *282*, 6823-6832.
Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408-411.

Tissenbaum, H. A., and Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature *410*, 227-230.

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell *79*, 1147-1156.

Tsang, A. W., and Escalante-Semerena, J. C. (1998). CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase activity in cobT mutants during cobalamin biosynthesis in Salmonella typhimurium LT2. J Biol Chem *273*, 31788-31794.

Ungerstedt, J. S., Sowa, Y., Xu, W. S., Shao, Y., Dokmanovic, M., Perez, G., Ngo, L., Holmgren, A., Jiang, X., and Marks, P. A. (2005). Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. Proc Natl Acad Sci U S A *102*, 673-678.

Van den Wyngaert, I., de Vries, W., Kremer, A., Neefs, J., Verhasselt, P., Luyten, W. H., and Kass, S. U. (2000). Cloning and characterization of human histone deacetylase 8. FEBS Lett *478*, 77-83.

van der Horst, A., Tertoolen, L. G., de Vries-Smits, L. M., Frye, R. A., Medema, R. H., and Burgering, B. M. (2004). FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem *279*, 28873-28879.

Van Lint, C., Emiliani, S., and Verdin, E. (1996). The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr *5*, 245-253.

Vannini, A., Volpari, C., Filocamo, G., Casavola, E. C., Brunetti, M., Renzoni, D., Chakravarty, P., Paolini, C., De Francesco, R., Gallinari, P.*, et al.* (2004). Crystal structure of a eukaryotic zinc-dependent histone deacetylase, human HDAC8, complexed with a hydroxamic acid inhibitor. Proc Natl Acad Sci U S A *101*, 15064-15069.

Vaquero, A., Scher, M., Erdjument-Bromage, H., Tempst, P., Serrano, L., and Reinberg, D. (2007). SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. Nature *450*, 440-444.

Vaquero, A., Scher, M., Lee, D., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004). Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. Mol Cell *16*, 93-105.

Vaquero, A., Scher, M. B., Lee, D. H., Sutton, A., Cheng, H. L., Alt, F. W., Serrano, L., Sternglanz, R., and Reinberg, D. (2006). SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. Genes Dev *20*, 1256-1261.

Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149-159.

Verdel, A., Curtet, S., Brocard, M. P., Rousseaux, S., Lemercier, C., Yoshida, M., and Khochbin, S. (2000). Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. Curr Biol *10*, 747-749.

Verdel, A., and Khochbin, S. (1999). Identification of a new family of higher eukaryotic histone deacetylases. Coordinate expression of differentiation-dependent chromatin modifiers. J Biol Chem *274*, 2440-2445.

Verdin, E. (2007). AROuSing SIRT1: identification of a novel endogenous SIRT1 activator. Mol Cell *28*, 354-356.

Verger, A., Perdomo, J., and Crossley, M. (2003). Modification with SUMO. A role in transcriptional regulation. EMBO Rep *4*, 137-142.

Vidali, G., Boffa, L. C., Bradbury, E. M., and Allfrey, V. G. (1978). Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. Proc Natl Acad Sci U S A *75*, 2239-2243.

Villagra, A., Cheng, F., Wang, H. W., Suarez, I., Glozak, M., Maurin, M., Nguyen, D., Wright, K. L., Atadja, P. W., Bhalla, K.*, et al.* (2009). The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nat Immunol *10*, 92-100.

Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res *55*, 5187-5190.

Wang, A. H., Kruhlak, M. J., Wu, J., Bertos, N. R., Vezmar, M., Posner, B. I., Bazett-Jones, D. P., and Yang, X. J. (2000). Regulation of histone deacetylase 4 by binding of 14-3-3 proteins. Mol Cell Biol *20*, 6904-6912.

Wang, A. H., and Yang, X. J. (2001). Histone deacetylase 4 possesses intrinsic nuclear import and export signals. Mol Cell Biol *21*, 5992-6005.

Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress, W. D., and Chen, J. (2006). Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. Nat Cell Biol *8*, 1025-1031.

Wang, F., Nguyen, M., Qin, F. X., and Tong, Q. (2007). SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. Aging Cell *6*, 505-514.

Wang, R. H., Sengupta, K., Li, C., Kim, H. S., Cao, L., Xiao, C., Kim, S., Xu, X., Zheng, Y., Chilton, B.*, et al.* (2008a). Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer Cell *14*, 312-323.

Wang, R. H., Zheng, Y., Kim, H. S., Xu, X., Cao, L., Luhasen, T., Lee, M. H., Xiao, C., Vassilopoulos, A., Chen, W.*, et al.* (2008b). Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis. Mol Cell *32*, 11-20.

Weindruch, R., Walford, R. L., Fligiel, S., and Guthrie, D. (1986). The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J Nutr *116*, 641-654.

Wen, Y. D., Perissi, V., Staszewski, L. M., Yang, W. M., Krones, A., Glass, C. K., Rosenfeld, M. G., and Seto, E. (2000). The histone deacetylase-3 complex contains nuclear receptor corepressors. Proc Natl Acad Sci U S A *97*, 7202-7207.

Westendorf, J. J., Zaidi, S. K., Cascino, J. E., Kahler, R., van Wijnen, A. J., Lian, J. B., Yoshida, M., Stein, G. S., and Li, X. (2002). Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. Mol Cell Biol *22*, 7982-7992.

Wojcik, M., Mac-Marcjanek, K., and Wozniak, L. A. (2009). Physiological and pathophysiological functions of SIRT1. Mini Rev Med Chem *9*, 386-394.

Wong, S., and Weber, J. D. (2007). Deacetylation of the retinoblastoma tumour suppressor protein by SIRT1. Biochem J *407*, 451-460.

Xu, W. S., Parmigiani, R. B., and Marks, P. A. (2007). Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene *26*, 5541-5552.

Yamasaki, L. (1999). Balancing proliferation and apoptosis in vivo: the Goldilocks theory of E2F/DP action. Biochim Biophys Acta *1423*, M9-15.

Yang, B., Zwaans, B. M., Eckersdorff, M., and Lombard, D. B. (2009). The sirtuin SIRT6 deacetylates H3 K56Ac in vivo to promote genomic stability. Cell Cycle *8*, 2662-2663.

Yang, W. M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996). Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc Natl Acad Sci U S A *93*, 12845-12850.

Yang, W. M., Tsai, S. C., Wen, Y. D., Fejer, G., and Seto, E. (2002). Functional domains of histone deacetylase-3. J Biol Chem *277*, 9447-9454.

Yang, W. M., Yao, Y. L., Sun, J. M., Davie, J. R., and Seto, E. (1997). Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. J Biol Chem *272*, 28001-28007.

Yang, X. J., and Gregoire, S. (2005). Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol *25*, 2873-2884.

Yang, X. J., and Seto, E. (2008). The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol *9*, 206-218.

Yang, Y., Fu, W., Chen, J., Olashaw, N., Zhang, X., Nicosia, S. V., Bhalla, K., and Bai, W. (2007). SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. Nat Cell Biol *9*, 1253-1262.

Yang, Y., Hou, H., Haller, E. M., Nicosia, S. V., and Bai, W. (2005). Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. EMBO J *24*, 1021-1032.

Yeung, F., Hoberg, J. E., Ramsey, C. S., Keller, M. D., Jones, D. R., Frye, R. A., and Mayo, M. W. (2004). Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J *23*, 2369-2380.

Yoshida, M., Horinouchi, S., and Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays *17*, 423-430.

You, A., Tong, J. K., Grozinger, C. M., and Schreiber, S. L. (2001). CoREST is an integral component of the CoREST- human histone deacetylase complex. Proc Natl Acad Sci U S A *98*, 1454-1458.

Yuan, J., Minter-Dykhouse, K., and Lou, Z. (2009). A c-Myc-SIRT1 feedback loop regulates cell growth and transformation. J Cell Biol *185*, 203-211.

Yuan, Z., and Seto, E. (2007). A functional link between SIRT1 deacetylase and NBS1 in DNA damage response. Cell Cycle *6*, 2869-2871.

Zhang, C. L., McKinsey, T. A., Lu, J. R., and Olson, E. N. (2001). Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. J Biol Chem *276*, 35-39.

Zhang, Q., Wang, S. Y., Fleuriel, C., Leprince, D., Rocheleau, J. V., Piston, D. W., and Goodman, R. H. (2007). Metabolic regulation of SIRT1 transcription via a HIC1:CtBP corepressor complex. Proc Natl Acad Sci U S A *104*, 829-833.

Zhang, X., Ozawa, Y., Lee, H., Wen, Y. D., Tan, T. H., Wadzinski, B. E., and Seto, E. (2005). Histone deacetylase 3 (HDAC3) activity is regulated by interaction with protein serine/threonine phosphatase 4. Genes Dev *19*, 827-839.

Zhao, W., Kruse, J. P., Tang, Y., Jung, S. Y., Qin, J., and Gu, W. (2008). Negative regulation of the deacetylase SIRT1 by DBC1. Nature *451*, 587-590.

Zhou, X., Marks, P. A., Rifkind, R. A., and Richon, V. M. (2001). Cloning and characterization of a histone deacetylase, HDAC9. Proc Natl Acad Sci U S A *98*, 10572-10577.

Zhou, X., Richon, V. M., Rifkind, R. A., and Marks, P. A. (2000a). Identification of a transcriptional repressor related to the noncatalytic domain of histone deacetylases 4 and 5. Proc Natl Acad Sci U S A *97*, 1056-1061.

Zhou, X., Richon, V. M., Wang, A. H., Yang, X. J., Rifkind, R. A., and Marks, P. A. (2000b). Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras. Proc Natl Acad Sci U S A *97*, 14329-14333.

Zhu, L. (2005). Tumour suppressor retinoblastoma protein Rb: a transcriptional regulator. Eur J Cancer *41*, 2415-2427.

Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K. P., and Gottlicher, M. (2004). Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell *5*, 455-463.

Zschoernig, B., and Mahlknecht, U. (2009). Carboxy-terminal phosphorylation of SIRT1 by protein kinase CK2. Biochem Biophys Res Commun *381*, 372-377.

About the Author

Neha Kabra attended the University of Delhi, India from 1999-2002 where she received her Bachelor of Science degree in Microbiology. She then joined the Genetics program at the University of Delhi where she earned her Master of Science degree in 2004. During this time she also worked as a summer intern in the lab of Dr. Veronica Rodriguez at the TATA Institute of Fundamental Research, Bombay, where she gained valuable experience in researching fly genetics. She also passed the CSIR-UGC National Eligibility Test for Lectureship in Life Sciences, India. In August 2004, she was accepted in the Cancer Biology Ph.D. program at the H. Lee Moffitt Cancer Center and Research Institute. Here she joined the lab of Dr. Jiandong Chen and completed her dissertation work in the field of Sirtuins. Her work aimed at studying the role of SIRT1 in cancer.