

**REGULATION OF TRANSLATION AND TRANSCRIPTION BY SIRT1: POTENTIAL
NOVEL MECHANISMS FOR REGULATING STRESS RESPONSE AND AGING**

by

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REGULATION OF TRANSCRIPTION AND TRANSLATION BY SIRT1: POTENTIAL NOVEL MECHANISMS FOR REGULATING STRESS RESPONSE AND AGING

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University of Pittsburgh, 2007

SIRT1 is a NAD⁺ dependent deacetylase that targets many histone and non histone proteins, thereby regulating a broad range of physiological processes such as metabolism, reproduction, development, and cell survival. In this study, we have identified novel binding partners for SIRT1 and documented three different cellular processes that are affected by these novel interactions.

Using a yeast two-hybrid screen, we have identified several potential binding partners for SIRT1. Transducin like Enhancer of split 1 (TLE1) and eIF2-alpha (eIF2 α) are two such proteins identified in the screen whose interaction with SIRT1 was further confirmed by co-immunoprecipitation.

TLE1 is co-repressor for several transcriptional factors including NF- κ B. We demonstrate that SIRT1 and TLE1 repress NF- κ B activity and that the catalytic activity of SIRT1 may not be critical for this. Using knock-out cell lines, we further demonstrate that both SIRT1 and TLE1 are required for the down-regulation of NF- κ B activity. Our results suggest that the interaction between SIRT1 and TLE1 is important for mediating repression of NF- κ B activity, potentially through a deacetylase independent mechanism.

SIRT1 protects cells from genotoxic and oxidative stress, whereas phosphorylation of eIF2 α is critical for translation attenuation and preferential expression of stress related genes under stress conditions. We demonstrate that SIRT1 depleted cells show higher levels of phosphorylated eIF2 α and delayed expression of the stress response protein, CHOP.

Furthermore, SIRT1 deficient cells show higher sensitivity to stress treatments and a delayed recovery of protein synthesis. SIRT1 associates with eIF2 α regardless of stress condition, SIRT1's catalytic activity or the phosphorylation state of eIF2 α . These observations suggest a novel aspect of SIRT1 mediated regulation of cellular stress response.

Both SIRT1 and the target of rapamycin (TOR) are involved in age related diseases and lifespan. We demonstrate for the first time that these two pathways are interconnected. We show that SIRT1 null mouse embryonic fibroblasts (MEFs) have larger cell morphology and upregulated mTOR signaling. Furthermore, SIRT1 activator reduces, whereas inhibitor activates the mTOR pathway. Rapamycin is effective in inhibiting mTOR activity in both SIRT1 positive and deficient cells. Finally, we show that SIRT1 physically associates with TSC2 in HeLa cells. These observations demonstrate that SIRT1 negatively regulates mTOR pathway upstream of mTOR complex-1 (TORC1), potentially, by regulating the TSC1/2 complex.

*Asato Ma Sat Gamaya
Tamaso Ma Jyotir Gamaya
Mrityor Ma Amritam Gamaya*

Lead me from the unreal to the real, lead me from darkness to light, lead me from death to immortality.

- Brhadaranyaka Upanishad (I.3.28)

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He, to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

- Albert Einstein

Dedicated to my parents

Rama and Aswini Kumar Ghosh

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THESIS OUTLINE

Chapter 1: This chapter consists of an introduction to the sirtuins in lower organisms and mammals, their various target substrates and their role in various cellular functions. This is followed by some background on the role of sirtuins in aging pathways, caloric restriction and insulin-signaling pathways. The human SIRT1 has been specifically discussed with regards to its role in metabolism, endocrine signaling and aging-related diseases such as cancer and neurodegeneration. In addition, the specific chemical and natural regulator compounds for SIRT1's catalytic activity have also been discussed in brief. This section gives a background of SIRT1's role in varied cellular pathways indicating the complexity that lie behind SIRT1's potential role in regulating mammalian aging.

Chapter 2: This chapter describes the identification of transducin like enhancer of split-1 (TLE1) as a novel binding partner for SIRT1, and its role in mediating SIRT1's regulation on NF- κ B activity.

Chapter 3: This chapter describes the identification of eIF2 α as yet another novel binding partner for SIRT1, and its potential role in regulating SIRT1 mediated cellular stress response. The role of SIRT1 in regulating eIF2 α demonstrates a novel role for SIRT1 in translation regulation and stress response.

Chapter 4: This chapter describes the role of SIRT1 in regulating the mammalian Target of Rapamycin (mTOR) pathway. This pathway is involved in cellular growth and size and has been implicated in longevity in lower organism. Elucidation of SIRT1 as a regulator of this pathway is thus relevant with regards to SIRT1's role in cancer and mammalian aging.

Chapter 5: This chapter recaps the major findings of this study, their relevance with regards to the biology of sirtuins, its role in mammalian longevity and potential future directions of research.

1.0 GENERAL INTRODUCTION

In the past five to six decades, understanding the aging process and the prospects of regulating longevity has captured the imagination of scientists all over the world. Although death is inevitable, understanding the process of aging and elucidating how and why cells, and eventually organisms, die can greatly help us understand and modulate many disease processes that occur with age, thereby enabling us to come up with useful interventions.

As early as the 1930's, scientists observed that restricted food intake, termed now as caloric restriction (CR), can increase lifespan in rodents. Since then, this phenomenon has been verified in various organisms from yeast to primates, indicating that the process of aging may be evolutionarily conserved. Discoveries in the past decade have revolutionized the field of aging research, leading to wide acceptance of the fact that aging, like most other biological processes, can be regulated. A novel class of regulators, namely, the silent information regulator (SIR2) family or sirtuins, has been shown to regulate aging and mediate CR-induced longevity in lower organisms such as yeast, worms and flies [189,191,256,294]. However, it is yet to be determined if sirtuins regulate longevity in mammals. In the past one decade genetic studies using model organisms have identified specific genes and signaling pathways that regulate cellular damage and senescence. These studies indicate a potential critical role for sirtuins in mammalian longevity. Cellular senescence was first described about four decades ago. The word 'Senescence' is derived from a Latin word *Senex*, meaning old man or old age. Although

scientifically the term ‘senescence’ was applied to cells that stopped dividing in culture [119], it can be interchangeably used with aging, since it describes the deteriorative processes in living organisms that can ultimately lead to death. The first molecular explanation for the limited capacity of cells to divide was provided by the discovery of telomere shortening [24,116], a process regulated by sirtuins. Since then, scientists have been diligently examining other causes that can lead to aging and death. Broadly, the regulators of lifespan that have been identified to date, can be categorized as 1) Caloric restriction 2) Gene Silencing 3) hormonal signals and 4) oxidative stress. In the past decade, the mammalian sirtuins have been implicated extensively in regulating all of the above processes, thereby affecting multiple signaling pathways that are interconnected in a very complex way. Although significant advances have been made to elucidate the various cellular processes that lead to aging phenotype, we are still far from understanding how these processes and pathways are coordinated in a coherent way to facilitate cellular struggle against damage and death. Continued research to understand more regarding the roles of specific genes and proteins involved in pathways regulating lifespan, will greatly help our search to find the secret of healthy, disease free and youthful additional years in our lives.

1.1 BACKGROUND ON SIRTUINS

In the past decade, a novel class of regulators called the ‘Silent information regulator 2 (Sir2) family or Sirtuins, have been implicated in regulating organism aging and lifespan. Initially discovered in yeast, Sirtuins are a family of proteins with protein deacetylase and ADP-ribosyltransferase activity [22,132,193]. The name ‘Sirtuins’ was first given to the family of these proteins by Roy Frye in 1991 who identified five of the human SIR2 homologues, SIRT1-5. The first sirtuin gene was discovered in *Saccharomyces cerevesiae* more than two decades ago by Klar and colleagues [163]. The founding member of the Sirtuin family of proteins, the yeast Sir2 (Sir2p), was originally known as MAR1, for mating-type regulator 1. It was discovered by observing a spontaneous mutation that caused sterility due to loss of silencing at the mating-type loci *HMR* and *HML*. Later on, a series of mutations resulting in sterile phenotypes were co-discovered by Jasper Rine, who named the set of four genes, *homologues of SIR2 (HST)*, responsible for this trait, as *Silent Information Regulator (SIR) 1-4* [137,163,276]. Subsequently, SIR2 homologues were found in bacteria, worms, flies, plants and mammals, suggesting that the Sirtuin family genes are ancient and evolutionarily conserved.

More than a decade after the discovery of the *SIR2* gene, two different groups [6,99], demonstrated that the *SIR2* gene from the Sirtuin family, is required to suppress rDNA recombination and silencing at telomeric DNA. Subsequently it was shown that the gene silencing at mating-type loci and telomeres, is associated with hypo-acetylated histone proteins at the N-terminal lysine residues [30]. After the initial discovery that sirtuins metabolize NAD and possess ADP-ribosyltransferase activity, it was soon established that the enzymatic activity of yeast Sir2 protein was essential for gene silencing [87,290]. The first identified protein substrates of the yeast Sir2 (Sir2p) were histones [22]. Initially it was thought that ADP-

ribosylation of histones by Sir2 interferes with histone acetylation, leading to hyper-acetylation and loss of silencing in Sir2 mutants. However, soon it was shown that Sir2 deacetylated histones and this activity was absolutely dependent on NAD⁺ [132,177]. The NAD⁺ dependent deacetylase activity was later described for numerous other sirtuins, including bacterial CobB, archaeobacterial SIR2-AF (*Archaeoglobus fulgidus*) and human SIRT1-3 and 5 [280]. Sirtuins were thus categorized as class III histone deacetylase (HDAC-III). While class I and II histone deacetylases use zinc as a co-factor [122], the sirtuins are NAD⁺ dependent in that they consume one NAD⁺ for removing each acetyl group from the protein substrate [22]. Landry et al [176] elucidated the mechanism of Sir2 mediated deacetylation showing that the hydrolysis of every molecule of NAD⁺ produces one molecule of nicotinamide and one molecule of 2'3'-O-acetyl-ADP-ribose (OAADPr), as shown in Figure 1.1.

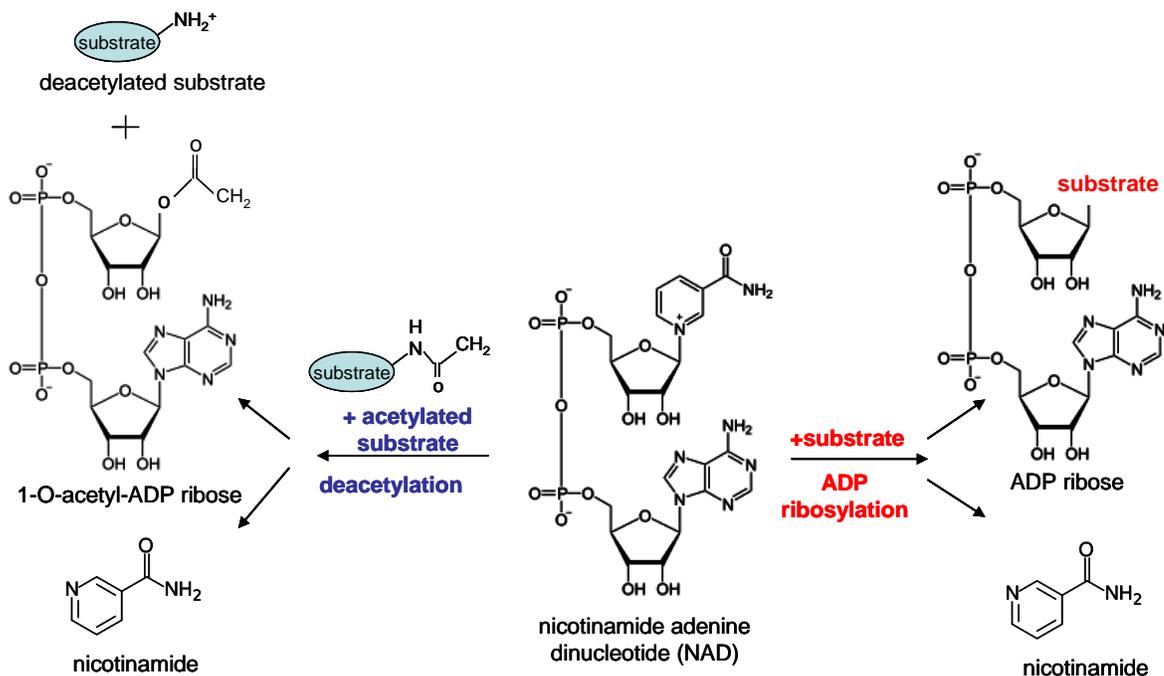


Figure 1.1 Sirtuin enzymatic activities.

SIRT1 acts as a NAD⁺ dependent deacetylase and mono-ADP-ribosyltransferase. Both deacetylation and ADP-ribosylation occur via cleavage of NAD⁺ to release nicotinamide. The ε-acetyl lysine residue of the target protein

serve as substrate for sirtuin deacetylation. NAD⁺ and acetylated protein are converted to free lysine side chain, nicotinamide (NAM) and 2'-O-acetyl-ADP-ribose (2'-OAADPr) by Sirt1. The by-product equilibrates in solution with 3'-O-acetyl-ADP-ribose.

OAADPr formed as a byproduct of NAD⁺ dependent deacetylation reaction acts as a secondary messenger for sirtuin triggered signaling pathways. The absolute requirement of NAD⁺ for sirtuin catalysis suggests that sirtuins may have evolved as sensors of cellular energy and redox states coupled to the metabolic status of the cell. Increasing evidence support that sirtuins are indeed adapted to interact with changes in NAD⁺ involving metabolic pathways, manifested by changes in the concentration of NAD⁺, NADH and/or nicotinamide [68,103,182,189-191,253,266]. Nicotinamide, and the reduced dinucleotide, NADH, are inhibitors of sirtuins. Unlike class I and II HDACs, sirtuins are not affected by Trichostatin A (TSA).

1.1.1 Mammalian sirtuins

The mammalian sirtuin family consists of seven sirtuins discovered in humans so far namely, SIRT1-7 [22,87]. All of these have an NAD⁺ -dependent catalytic core domain, which may act preferentially as a NAD⁺ -dependent deacetylase (DAC) and/or mono-ADP-ribosyl transferase (ART). The N-terminal and C-terminal sequences that flank the catalytic core domain vary in length between the different sirtuins. The seven mammalian sirtuins also differ in their sub-cellular localization; SIRT1, SIRT6 and SIRT7 being predominately nuclear, SIRT2 cytoplasmic [86,224] and SIRT3, SIRT4 and SIRT5 mostly described as mitochondrial. Although initially,

SIRT1 was known to be a nuclear protein, more recently it has been found to shuttle between the nucleus and the cytoplasm, displaying some important cytoplasmic functions as well [55,110,217,289].

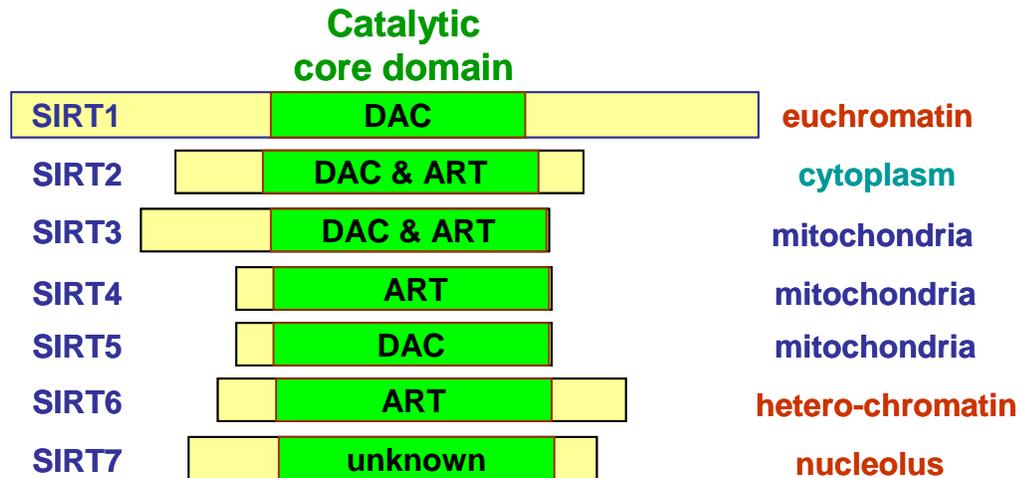


Figure 1.2 Mammalian Sirtuins

The core catalytic domain is conserved between all the seven mammalian sirtuins, SIRT1-7. The N and C-terminal flanking sequences vary in length. The sub-cellular localization also varies among the seven sirtuins.

In the nucleus, a large fraction of SIRT1 is associated with euchromatin, whereas SIRT6 associates with the heterochromatin and SIRT7 localizes in the nucleolus [81,211]. Among the seven sirtuins, SIRT1 show robust deacetylase activity [224,302], SIRT5 has weak deacetylase activity and SIRT2 and 3 possess both deacetylase as well as mono-ADP-ribosyl transferase activities [86,224,273]. SIRT4 and 6, on the other hand, are mono-ADP-ribosyl transferases [109,193], and no significant activity has been found for SIRT7 yet. Like their diverse sub-cellular localization, the mammalian sirtuins are expressed differentially in organs, have multiple target substrates and affect a broad range of cellular functions.

Table 1.1 Expression pattern, cellular distribution and functions of mammalian sirtuins

Gene	High expression levels	Low expression levels	Sub-cellular localization	Target proteins
SIRT1	Brain, testis, skeletal muscle, kidney (+++), thymus, uterus (++)	Liver, spleen, lungs, ovary, bone marrow, heart	nuclear	P53, Ku70, NF- κ B, PGC1 α , MEF2D, MyoD, PPAR γ , FOXO, p300, AceCS1, tat
SIRT2	Brain, skeletal muscle (+++), liver, testis, kidney, heart (++)	Thymus, lungs, bone marrow, uterus, ovary, spleen	cytoplasmic	α -tubulin
SIRT3	Ovary (+++), most other organs (++)	-	mitochondrial	PGC1 α , AceCS2
SIRT4	Brain, testis, heart, lungs (+++), liver, skeletal muscle, kidney, thymus, uterus, ovary (++)	Bone marrow, spleen	mitochondrial	Glutamate dehydrogenase
SIRT5	Brain, testis, skeletal muscle, kidney, heart (+++), liver, ovary, lungs, thymus, uterus, bone marrow (++)	spleen	mitochondrial	unknown
SIRT6	Fetal brain (+++), brain, liver, testis, skeletal muscle, kidney, heart, ovary (++)	Spleen, thymus, uterus, bone marrow, lungs	nuclear	DNA pol β
SIRT7	Brain, testis, kidney, spleen, liver	Other organs	nuclear	RNA pol I

Of the seven mammalian sirtuins, SIRT1 is the most extensively studied, with more than a dozen known substrates and implicated roles in a wide range of cellular processes including cell survival and apoptotic pathways.

1.1.2 Functions of mammalian sirtuins

Gene Expression

Repression of gene expression is associated with histone hypo-acetylation. Heterochromatin, which is the more tightly packed form of chromatin, is associated with hypo-acetylated histones. SIRT1, like the yeast Sir2, facilitates the formation of heterochromatin by targeting and deacetylating various histone proteins. It deacetylates histone protein H1 at the lysine residues 9 and 26, H3 at 14 and H4 at 16. Apart from deacetylating histone proteins, SIRT1 plays a role in gene expression by targeting transcription factors. The numerous non-histone targets of SIRT1 include TAF₆₈ [TBP (TATA-box binding protein) associated factor I 68], p300, PACF [p300/cAMP-response-element-binding protein-associated factor], GCN5, MyoD, MEF2 (MADS box transcription factor enhancer factor 2), p19^{ARF}, p53, HIC1, NF-κB, PGC1α, PPARγ, aP2, FOXO1,3a and 4, E2F1, p73, BCA3, Hes1 and Hey2, BCL11A, CTIP2, NCoR, SMRT, UCP2, HIV-Tat [11,27,28,34,48,49,53,55,62,81,89,91,93,166,198,216,218,219,233,241,270,271,282,288,298,299,302,305,320,327].

TAF₆₈ is a component of the TBP containing complex, TIF (transcription initiation factor)-IB/SL, which regulates transcription by RNA Pol I (RNA polymerase I). SIRT1 deacetylates TAF₆₈, thereby decreasing its DNA binding activity, leading to repression of RNA Pol I-mediated transcription in vitro [218]. SIRT1 binds and deacetylates the acetyltransferase p300 at lysine 1020 and 1024, thereby inhibiting it. Since, p300 is a limiting transcription cofactor, its inhibition plays an important role in orchestrating cell differentiation and metabolism. The functions of the other targets of SIRT1 will be discussed in the following sections.

Several studies implicate SIRT1 in epigenetic gene regulation in cancer cells. Being identified as a component of the polycomb repressive complex 4 (PRC4), which harbors the SET

domain histone methyltransferase Ezh2 [172], SIRT1 regulates the expression of PRC4 target genes. It is believed that PRC4-mediated histone modifications may contribute to cancer specific epigenetic changes. Furthermore, in the context of epigenetic modifications of DNA, SIRT1 also deacetylates histone H1-K26 which helps spreading hypo-methylated histone H3-K79 and help form heterochromatin formation [299]. SIRT1 depleted mammalian cells show a reduction in H3 tri-MeK9, H4-MeK20 and H4-K16 hyperacetylation [132]. Interestingly, various tumors and tumor-derived cell lines show loss of H4-K16 acetylation and H4-K20 trimethylation, suggesting that these could be hallmarks of epigenetic modification in cancer cells [83]. Notably, SIRT1 localizes specifically to the promoters of tumor suppressor genes whose DNA is hypermethylated and silenced in many cancers.

Apoptosis and cell survival

SIRT1 plays a role in apoptosis by targeting multiple proteins such as p53, p73, E2F, HIC1 and Ku70. SIRT1 binds the tumor suppressor p53 and deacetylates it at multiple lysine residues, thereby inhibiting p53 transactivation and suppressing apoptosis in response to oxidative stress and DNA damage [198,302]. SIRT1 also binds HIC1 (hypermethylated in cancer 1) transcriptional repressor and mediates the bypass of apoptosis, potentially by promoting cell survival and tumorigenesis via p53. Since HIC1 can repress SIRT1 expression and p53 is able to transactivate HIC1 transcription, SIRT1, HIC1 and p53 are believed to act in a complex loop where HIC1 represses SIRT1, promoting p53 activity and apoptosis under stress. However, under conditions, where cells are to be recovered from DNA damage, p53 down-regulates HIC1, which induces SIRT1 transcription and promotes cell survival.

Another mechanism by which SIRT1 regulates apoptosis is by binding and deacetylating the DNA repair factor Ku70. Ku70 acts as an inhibitor of Bax mediated apoptosis. Deacetylated Ku70 complexes with the proapoptotic factor Bax, sequestering it away from mitochondria, thereby blocking it from triggering apoptosis in 293 cells in response to stress [55]. SIRT1 also binds the cell proliferation and cell-cycle regulator, E2F1, and inhibits the apoptotic function of E2F1. On the other hand, E2F1 binds directly to the SIRT1 promoter and induces its transactivation, forming a negative feedback loop between SIRT1 and E2F1 functions. This mutual regulation of SIRT1 and E2F1 protects against DNA damage [305]. p73 is yet another protein that SIRT1 targets to regulate apoptosis. Similar to p53, SIRT1 binds and deacetylates p73, thereby suppressing its transcriptional activity and inhibiting p73 mediated apoptosis in 293 cells [62].

Stress resistance and cell survival

SIRT1 also plays a role in cell survival by regulating the Forkhead transcription factors (FOXOs). SIRT1 has been documented to deacetylate three out of the four known FOXO proteins, namely Foxo1, Foxo3a and Foxo4. SIRT1 affects Foxo3a in neurons and fibroblasts, reducing stress induced apoptosis and increasing expression of DNA repair and cell cycle checkpoint genes [34,216]. SIRT1 also deacetylates Foxo4 and rescues its repression under oxidative stress, thereby increasing expression of growth arrest and DNA repair protein, GADD45 (growth arrest and DNA-damage-inducible α) [166,298]. Acting through Foxo4, SIRT1 also suppresses the pro-apoptotic proteases caspase-3 and 7 only in transformed cells but not in normal cells [82]. Interestingly, caspase-9 and Bcl-xL regulate SIRT1 cleavage during apoptosis, shifting its localization from nucleus to cytoplasm [229].

Furthermore, SIRT1 protects pancreatic β -cells against glucose induced cytotoxicity by acting through FOXO1 [162]. In diabetic patients, chronically high plasma glucose causes cytotoxicity leading to β -cell degeneration. This is believed to be caused by increased mitochondrial oxidation rates, due to higher glucose levels, leading to increased ROS production. Under these conditions, SIRT1 is required in sustaining FOXO1-mediated transcription of MafA and NeuroD, which regulate expression of insulin gene2 to prevent apoptosis.

Cellular Senescence

Cellular senescence is a state of permanent cell cycle arrest which is manifested by defined morphological changes. Senescence can be natural or induced by certain stimuli. SIRT1's role in regulating cellular senescence is conflicting. Under certain conditions, SIRT1 has been found to localize with PML (promyelocytic leukemia) in discrete nuclear structures called the nuclear bodies. PML proteins are believed to act as co-activator or co-repressor to various transcription factors affecting apoptotic signals. It is believed that SIRT1 rescues primary mouse embryonic fibroblasts from PML-mediated premature cellular senescence by inhibiting the pro-apoptotic factor p53 [178]. However, in other cases, SIRT1 has been shown to promote cellular senescence, SIRT1 null MEFs show extended replicative potential and higher proliferation during chronic but sublethal stress [53]. Interestingly, lower SIRT1 levels have been documented in dividing tissue of older mice, such as testis and thymus or in cells that have been serially passaged. However, this was not true for immortalized cells or post-mitotic organs [265]. Thus, although SIRT1's regulatory role in senescence is conflicting, SIRT1 mediated regulation of senescence may play a significant role with regard to tumorigenesis in the elderly and aging.

DNA Repair

A couple of reports have suggested a potential role of the mammalian sirtuins in DNA repair. Recently, Mostoslavsky *et al* showed that SIRT6 knockout mice exhibit impairment in base excision repair [214]. While the mechanism by which SIRT6 regulates DNA repair is not clear, the various phenotypic defects found in SIRT6 knockout mice such as premature aging, abnormal spine curvature and metabolic defects have been attributed to defects in DNA repair. More recently, SIRT1 has also been implicated in DNA repair mechanism. Upon exposure to radiation, SIRT1 enhances DNA repair capacity and deacetylation of the repair protein Ku70. Over-expression of SIRT1 results in the increased repair of DNA strand breakages produced by radiation. On the other hand, repression of endogenous SIRT1 expression by SIRT1 siRNA decreases repair activity, indicating that SIRT1 can regulate DNA repair capacity of cells with DNA strand breaks [140].

Inflammation

SIRT1 plays a role in inflammation by regulating a key regulator NF- κ B. SIRT1 represses NF- κ B activity possibly by multiple mechanisms. It has been shown that SIRT1 deacetylates the RelA/p65 subunit of NF- κ B, thereby inhibiting its transactivation potential [320]. Consistent with this, it has been shown that cigarette smoke extracts can increase pro-inflammatory responses mediated by NF- κ B by decreasing the interaction between SIRT1 and RelA/p65 resulting in increased acetylation and activation of NF- κ B [314]. Interestingly, SIRT1 is expressed at higher levels in calorie restricted rodents which also show decreased inflammatory responses. Recently in a high-throughput screen, SIRT1 activating compounds were shown to

have anti-inflammatory properties such as reduction of the pro-inflammatory cytokine ‘tumor necrosis factor (TNF- α)’ [221].

Development

Many lines of evidence suggest a role of SIRT1 in development. Using a Sir2 knock out transgenic mice model, McBurney *et al.* showed that the protein SIRT1 is important for embryogenesis and gametogenesis. First, only half of the typically expected numbers of pups are born, and of those that are born, only 20% survive to adulthood. These mice showed developmental defects such as markedly smaller size as compared to their littermates, slower development, defects in eye morphogenesis and cardiac septation. Furthermore, the mice that survive to adulthood are sterile in both sexes, with males having lower sperm count and females failing to ovulate, potentially due to hormonal inefficiency [206]. The developmental defects in SIRT1 mutant mice can be explained by SIRT1’s regulation of the transcriptional repressors Hes1 and Hey2, which play a role in development [288]. Apart from this, SIRT1 is also known to regulate BCL11A and CTIP2, a mammalian and chicken protein respectively, which play a role in haematopoietic cell development and malignancies [271]. Consistent with SIRT1’s role in development, another report showed that SIRT1 is expressed at higher levels in the heart, brain, spinal cord and dorsal root ganglia of embryos [262]. Another sirtuin member, SIRT2, has been shown to interact with an homeobox transcription factor important for embryogenesis, HOXA10, indicating potential role for the other sirtuins in development [11].

Reproduction

It has been documented that developing spermatocytes express higher levels of SIRT1 and deletion of the SIRT1 gene leads to severe sperm abnormality and sterility in mice [206]. Thus SIRT1 potentially plays a role in the reproductive capacity of animals.

Muscle maintenance

SIRT1 regulates muscle differentiation and muscle mass maintenance by regulating multiple proteins. SIRT1 deacetylates and inhibits the transcription factor MyoD, a key player in muscle differentiation. On the other hand, SIRT1's activity is regulated by decreasing levels of NAD⁺ during muscle differentiation to alleviate SIRT1-mediated MyoD suppression [89]. SIRT1 also deacetylates myocyte enhancing factor 2 (MEF2), another transcription factor that regulate muscle differentiation. Deacetylation of MEF2 by SIRT1 facilitates the HDAC4 mediated SUMO addition by E3 ligase on MEF2, thereby inhibiting MEF2 mediated transcription [327].

Reduction in muscle mass is a common cause in diseases like muscular dystrophy, cancer and aging. Balanced turnover of protein is critical for muscle mass maintenance. Two proteins involved in proteasome mediated proteolysis are MnRF1 (for muscle RING finger 1) and MAFbx/atrogen-1 (for muscle atrophy F-box) [98]. These two proteins are transcriptionally regulated by NF- κ B and FOXO pathways, which in turn are regulated by SIRT1. Thus, along with its regulation of MyoD, MEF2, NF- κ B and Forkhead transcription factors, SIRT1 plays a broader role in controlling muscle mass maintenance during injury and aging.

Metabolism

The first evidence that suggested a potential role of SIRT1 in metabolism was the induction of SIRT1 in fasting tissues such as the brain, fat, kidney, muscle and liver [55]. Three different mammalian sirtuins play a role in regulating metabolism; namely SIRT1, SIRT3 and SIRT4. First, SIRT1 plays a role in the regulation of hepatic gluconeogenesis by potentiating FOXO1 activity in hepatocytes to direct glucose metabolism towards gluconeogenesis [84]. In addition SIRT1 interacts with and deacetylates PGC1- α , thereby repressing glycolysis and promoting gluconeogenic gene expression. Furthermore, increased NAD⁺ and pyruvate levels in fasting liver results in SIRT1 mediated upregulation of PGC1- α [255]. By regulating PGC1- α , SIRT1 also regulates mitochondrial function and metabolic homeostasis resulting in increased oxygen consumption in muscle fibers and induced oxidative phosphorylation and mitochondrial biogenesis [174,255].

SIRT3 increases cellular respiration and production of reactive oxygen species by decreasing mitochondrial membrane potential [273]. SIRT3 also induces mitochondrial genes such as PGC1- α , UCP2 and COX II and IV and ATP synthetase. In mammals, acetate from diet or endogenous reactions is converted to acetyl CoA in the cytoplasm by AceCS1 (acetyl-CoA synthetase) and in the mitochondria by AceCS2. AcetylCoA is a small molecule that is critical in fatty acids and amino acids synthesis. It has been documented that SIRT1 and SIRT3 deacetylate AceCS1 and 2, respectively, thereby activating these enzymes [110,269]. Yet another sirtuin, SIRT4, has been shown to regulate amino-acid stimulated insulin secretion in pancreatic β -cells [109]. SIRT4 also regulates glutamate dehydrogenase (GDH) by mono-ADP-ribosylation, thereby inhibiting GDH and slowing the conversion of glutamate into α -ketoglutarate.

The up-regulation of SIRT1 in response to fasting in white adipose tissue (WAT) suggested that SIRT1 might be able to regulate fat synthesis and/or release. Soon it was shown

that SIRT1 interacts with PPAR- γ and plays a role in fat metabolism by regulating adipogenesis and fat storage [241]. Furthermore, SIRT1 also regulates the levels of the adipocyte derived hormone adiponectin by acting through FOXO1 [247].

1.1.3 Sirtuins in aging pathways

One of the early events in the discovery of SIRT1 as a lifespan regulator was a genetic screen in yeast that showed that SIRT1 (yeast SIR2) is a determinant of yeast replicative lifespan, which measures the number of cell division a mother cells can undergo [155]. Later on it was shown that increasing dosage of SIRT1 gene extends lifespan, whereas inactivation of SIR2 leads to shortening of lifespan [149]. In yeast, accumulation of extra-chromosomal rDNA circles (ERCs) leading to enlarged nucleoli, cell cycle arrest and cellular senescence are considered to be the cause of aging [278]. SIR2 represses recombination at the rDNA locus and prevents generation of ERCs, thus slowing down the aging process [149].

The regulation of lifespan by SIRT1 has been shown to be conserved in a wide range of organisms. In *C.elegans*, the worm SIRT1 homolog, SIR2.1, regulates life span through a pathway that is dependent on DAF16 (dauer formation protein 16), a Foxo protein homolog in worms which is a downstream target of the insulin/IGF pathway. *C.elegans* carrying a chromosome duplication containing SIR2.1 exhibit up to a 50% increase in lifespan [294]. However, the worm SIR2.1 does not regulate the insulin/IGF-1 pathway. Instead SIR2.1 associates with a class of scaffolding proteins, 14-3-3, which direct SIRT1's interaction with DAF16 and transcriptional activator of DAF16 target genes in a insulin/IGF-independent manner under stress conditions, suggesting multiple pathways by which SIR2.1 may regulate lifespan in worms [308]. Similarly, in the fly, *Drosophila melanogaster*, an extra copy of the dSir2 gene

causes the flies to live approximately 18-29% longer [256]. SIR2 is believed to mediate longevity in flies through a pathway related to caloric restriction.

While a direct role of the mammalian sirtuins in increasing longevity has not yet been shown, a growing list of evidence suggests that sirtuins may be evolutionarily conserved as lifespan regulators in mammals too. Initially, the mammalian sirtuins were believed to play a role in aging by regulating oxidative and genotoxic stress. However, increasing evidence suggest that, in the complex mammalian system, sirtuins may affect aging by regulating multiple independent processes such as glucose homeostasis, insulin secretion, fat metabolism and stress resistance [27,34,55,198,216,217,241,255,302,305]. Some recent reports have attempted to examine the direct effects of SIRT1 in mammalian tissue and organ longevity. Using transgenic mice with heart-specific over-expression of SIRT1, it was shown that mild to moderate expression of SIRT1 retards aging of the heart and induces resistance to oxidative stress and apoptosis, suggesting that SIRT1 could retard aging and confer stress resistance to the heart in vivo at low to moderate doses [2]. Caloric restriction and leanness is associated with increased longevity in organisms ranging from yeast to mammals indicating the role of adipose tissue in lifespan. It has been documented that decreased adiposity in fat specific insulin receptor knock out (FIRKO) mice leads to increased lifespan. Interestingly, SIRT1 plays a critical role in fat storage and adipogenesis in mammals, thereby linking adipose regulation of longevity to SIRT1 [241,247]. In the last couple of years, a direct role of SIRT1 in regulating the aging phenotype has emerged. First, it was shown that DeltaNp63alpha-mediated down-regulation of SIRT1 results in accelerated aging phenotype in the mouse [283]. Soon after, SIRT1 was shown to modulate premature senescence-like phenotype in human endothelial cells [231]. Resveratrol, an activator of SIRT1 was shown to improve health and survival of mice on high calorie diet [13]. Also,

SIRT1 has been documented to modulate insulin like growth factor (IGF) signaling in mice by increasing expression of the IGF binding protein IGFBP1, a secreted modulator of IGF function. This is interesting because the insulin/IGF pathway has been documented to play a role in lifespan in worms [183]. Thus, even though the exact mechanism by which SIRT1 may regulate lifespan in mammals is still not clear, a growing list of evidence indicates an extensive and direct participation of SIRT1 in the aging pathways in mammals.

1.1.4 Sirtuins in caloric restriction

Caloric restriction (CR) is a dietary regime in which an organism is provided with at least 20% fewer calories than it would naturally consume *ad libitum*, while maintaining adequate nutrition [207]. CR has been shown to increase the lifespan of a very wide range of organisms such as: yeast, fruit flies, worms, crustaceans, spiders and rodents [10,134,146,175,200,310]. The role of CR in lifespan in such a diverse group of organisms suggests that the mechanism by which CR regulates longevity is an ancient and evolutionarily well conserved pathway. CR has been shown to increase longevity by retarding age-related deterioration such as insulin resistance, collagen inelasticity, and reduced immune and neurological functions, thereby delaying onset of age-related diseases, such as metabolic syndrome, cancer, auto-immune and neurological disorders [12]. Evidence now suggests that CR may show age-related benefits in primates indicating its potential role in lifespan extension by reducing aging symptoms [204]. SIRT1 dependence on NAD⁺ for its catalytic activity makes SIRT1 a potential player in mediating CR responses to lifespan. The role of sirtuins in regulating CR was first demonstrated in the yeast *Saccharomyces cerevisiae* where lifespan extension by moderate CR (0.5% compared to normal 2%) was abrogated in strains that were mutated in the SIR2 or NPT1 (nicotinate

phosphoribosyltransferase 1) gene [189]. However, while SIR2 was required for lifespan extension in response to moderate CR, under severe CR (0.05 % glucose), lifespan extension is believed to be regulated by the TOR (target of rapamycin) pathway [150].

The SIR2 orthologs in *C. elegans* and *D. melanogaster* are involved in CR-induced lifespan extension in these organisms [256,308]. In mammals, CR elicits a complex set of physiological changes that are linked to healthier and longer lifespan, such as lower blood glucose, triglycerides, cholesterol and insulin levels, lower body fat, decreased body weight and increased physical activity along with increased insulin sensitivity and glucose tolerance [168]. Evidences suggest that most of these changes are potentially regulated by mechanisms in which SIRT1 plays a role. For example, SIRT1 knockout mice fail to show increased physical activity, even though they show some metabolic changes triggered by CR diet [42]. Secondly, SIRT1 over-expression in adipocytes results in decreased adipogenesis whereas knocking down SIRT1 enhances it [241]. Also, β -cell specific SIRT1-overexpressing transgenic mice show improved glucose tolerance and enhanced insulin secretion in response to glucose challenge [217]. Furthermore, SIRT1 regulates gluconeogenesis by deacetylating and activating PGC1- α .

Most recently, experiments using SIRT1 over-expressing transgenic mice displayed some phenotypes similar to mice on a calorie-restricted diet such as leaner body mass than littermate controls, higher metabolic activity, reduced blood cholesterol, adipokines, insulin and fasted glucose levels and higher glucose tolerance. Furthermore, transgenic mice performed better on a rotarod challenge and also showed a delay in reproduction. These findings suggest that increased expression of SIRT1 in mice elicits beneficial phenotypes that may be relevant to human health and longevity [26]. Although the direct role of SIRT1 in CR mediated lifespan extension has not

yet been proven in mammals, its involvement in fat storage, glucose homeostasis and insulin secretion places SIRT1 as a potential central regulator of CR mediated longevity in mammals.

1.1.5 Sirtuins in insulin / IGF-signaling

In the past decade, genetic analysis has revealed a lot about the pathways involved in aging. One of the better characterized pathways that have emerged to be a prominent player in aging is the Insulin/Insulin-like growth factor (IGF-1) signaling pathway. A single gene mutation in the IGF-signaling pathway led to increased lifespan extension in worms, flies and mice, indicating an evolutionarily conserved hormonal regulation of the aging process. The insulin/IGF-1 signaling pathway is nutrient activated and decreased signaling through this pathway has been shown to increase lifespan in *C.elegans* and mice [1,156,157]. Interestingly, sirtuins have shown an evolutionarily conserved role in modulating insulin signaling. The SIRT1 orthologs in worms and mice have been shown to modulate insulin/IGF-1 signaling by interacting through the Forkhead proteins, DAF16 and FOXO in *C.elegans* and mice, respectively.

In *C.elegans*, the SIRT1 ortholog, Sir2.1 extends worm lifespan through the forkhead transcription factor Daf-16 (homolog of mammalian FOXO) pathway. Loss of function of the Daf2 (insulin receptor substrate, IRS, in mammals) or AGE1 (phosphatidylinositol-3-kinase, PI3-K, in mammals) has been shown to extend the worm lifespan [160,213]. The long lived mutants require Daf-16 to be in the nucleus to activate target gene expression necessary for dauer formation (a larval developmental state of growth arrest induced upon food limitation). Activation of Daf2/AGE1 signaling phosphorylates and activates Akt, which then sequesters Daf16 in the cytoplasm resulting in its inactivation [188,228]. The Sir2.1 duplication that results in extended worm lifespan fails to do so in strains with mutated Daf-16. In addition, Sir2.1

duplicated strains do not further increase lifespan of the Daf-2 mutants, indicating that extra copies of Sir2.1 promote longevity through the Daf-2/Daf-16 pathway [294]. Recently, it has been documented that Sir2.1 physically interacts with Daf-16, and the scaffold protein 14-3-3 facilitates this interaction [17,307], emphasizing Sir2.1's role in worm insulin/IGF-1 signaling pathway.

The drosophila ortholog of SIRT1, dSir2, is also believed to play a role in the fly lifespan extension by regulating the insulin/IGF-1 pathway. It has been shown that dSir2 mutant flies can no longer show extended lifespan under calorie-restriction (CR), indicating that Sir2 extends fly longevity by acting through CR. Interestingly, the Drosophila histone deacetylase, Rpd3, which negatively regulates dSir2 expression is down-regulated by CR. Also, long-lived Rpd3 mutant flies show an increased dSir2 expression. Furthermore, flies deficient in CHICO, the drosophila homologue of the mammalian insulin receptor substrate (IRS), show a significant increase in lifespan. Recent findings have shown that activation of dFOXO in fly brain or fat body extends longevity and inhibits the endogenous insulin-dependent signaling in the fat body [54,96,130,257]. Thus, CR and insulin signaling have both been implicated in fly lifespan extension. Although dSir2 plays a role in CR-mediated lifespan in flies, its role in the insulin/IGF-1 signaling pathway potentially through dFOXO has not yet been proven directly.

The role of insulin/IGF-1 signaling pathway in extending mammalian lifespan has been studied in insulin-like growth factor receptor deficient (IGF-R +/-) mice. Unlike IGF-R-/- mice, which did not survive, IGF-R +/- mice with reduced IGF-R levels, showed longer lifespan more resistance to oxidative stress than IGF-R+/+ littermates. The long lived IGF-R +/- mice also exhibited low Akt kinase activity suggesting an increase in FOXO activity similar to the Daf-16 mediated longevity pathway in *C.elegans*. Furthermore, mice with inactivated insulin receptor in

adipose tissue also live longer and are protected against age-related obesity and subsequent metabolic abnormalities [23,126,241,255].

The role of mammalian SIRT1 in regulating the insulin/IGF-1 pathway is believed to be through the FOXO protein, which is regulated by deacetylation by SIRT1. Reduced insulin signaling increased FOXO1 activity and lifespan in mice [1,90,183]. SIRT1 and FOXO1 have been shown to physically interact. It has been demonstrated that SIRT1 can deacetylate FOXO1 in vivo, thereby either reducing or potentiating FOXO1 transcriptional activity depending on the context, as determined by its effects on the genes encoding IGF binding protein-1 and phosphoenolpyruvate carboxykinase [63,84,90,183]. Fasting (or CR) and resveratrol are known to increase SIRT1 activity, which then modulates FOXO activity, thereby mediating insulin signaling dependent effects on aging. Interestingly, Cohen *et al.* have demonstrated that CR-induced SIRT1 activation could be attenuated by IGF-1, suggesting why a reduction or defect in IGF-1 signaling could be beneficial to cell survival and lifespan extension [55]. Recently, it has been shown that treatment of rat hepatoma cells and HEK 293 cells with either insulin or IGF-1 lowers SIRT1 levels and the effects of these two ligands are additive. These results suggest that insulin/IGF-1 signaling can potentially oppose the cell survival effect of SIRT1, activated in the aging related metabolic context of caloric restriction.

1.2 ROLE OF SIRT1 IN REGULATING MAMMALIAN AGING

While SIRT1 orthologs in lower organisms have been shown to play a direct role in longevity, whether or not sirtuins regulate human longevity has not yet been proven. The cellular model for aging, which assays cellular senescence as a manifestation of aging, has shown a positive role of

SIRT1 in regulating mammalian aging. Senescence is a process in which cells undergo permanent cell-cycle arrest and characteristic morphological changes. p53, which is deacetylated and inactivated by SIRT1, plays a pivotal role in cellular senescence, thereby indicating a positive role of SIRT1 in decelerating cellular senescence [198,302]. Consistent with this, over-expression of SIRT1 in mouse embryonic fibroblasts (MEFs) inhibits p53 acetylation and premature senescence induced by promyelocytic leukemia protein (PML) oncogene [178]. Also, SIRT1 inhibitor, sirtinol has been shown to trigger senescence in human cancer cells [232]. Paradoxically, SIRT1 has also been shown to induce replicative senescence as shown in SIRT1 deleted MEFs that show extended replicative lifespan due to failure to upregulate p19(ARF) and p53 in response to chronic oxidative stress [53]. These observations suggest that SIRT1 may regulate senescence differently depending on stress conditions and inducing agents. Recently, SIRT1 has been shown to protect against stress-induced senescence-like phenotype in human endothelial cells by acting through p53 [231]. Also, down-regulation of SIRT1 was shown to accelerate an aging-like phenotype in mouse [283]. The other positive role of SIRT1 in promoting longevity in the cellular model for aging has been shown to be through the Forkhead proteins. SIRT1 physically binds and deacetylates the FOXO proteins thereby regulating their activity. It has been shown that SIRT1's regulation is required for FOXO mediated cellular stress response to protect cells from oxidative insults.

Apart from SIRT1's role in cellular model of aging by regulating stress resistance, growing evidence suggest SIRT1's role in mammalian aging at a systemic level. The critical evidences that implicate SIRT1 in mammalian aging pertain to SIRT1's role in metabolic regulation and protection against aging-related diseases such as cancer, diabetes and neurological disorders.

1.2.1 SIRT1 and glucose metabolism

Glucose metabolism is a major regulator of cellular energy state and a critical component of the physiology of CR. Organism with CR mediated longevity show increased insulin sensitivity and corresponding reduction in blood glucose and insulin levels. Up-regulation of SIRT1 in CR or fasting mammalian tissues is not surprising as SIRT1 has been shown to regulate glucose homeostasis by multiple mechanisms in two different tissues, namely insulin secretion in the pancreas and glucose metabolism in the liver.

Two independent studies have implicated a role for SIRT1 in regulation of insulin secretion in the pancreas. SIRT1 is constitutively expressed in the endocrine cells of the islets of Langerhans. Moynihan *et al.* have shown using a β -cell specific SIRT1 over-expressing (BESTO) mice that these animals have increased glucose tolerance as compared to wild type animals [27,217]. The BESTO mice showed increased insulin secretion in response to glucose and conversely, SIRT1^{-/-} mice or their isolated islets showed blunted insulin secretion. Also, they showed that SIRT1 binds to the promoter and represses transcription of the gene encoding the mitochondrial uncoupling protein UCP2. UCP2 uncouples mitochondrial respiration from ATP production and reduces the ability of β -cells to convert glucose to ATP[40]. Thus, by blocking UCP2 function, SIRT1 promotes efficient energy generation. Consistent with this, BESTO mice show higher ATP production while islets from SIRT1 knock out mice do not show any increase in the ATP levels in response to glucose. In starved wild-type mice, UCP2 protein levels increased in the pancreas compared to *ad libitum* fed animals [27]. Interestingly, decreased SIRT1 activity due to decreased NAD⁺ production in fasting pancreas could increase

UCP2 transcription. This suggests that NAD⁺ metabolism may provide a regulatory role for modulating insulin secretion in β -cells and glucose metabolism.

Another organ important in maintaining glucose homeostasis in response to changing nutrient conditions is the liver. During fasting, liver induces gluconeogenesis to supply other tissues with glucose. Several recent reports have provided evidence that suggest that this nutrient response in the liver is under the tight control of SIRT1. SIRT1 has been shown to regulate gluconeogenesis in liver by two different mechanisms, one involving the FOXO protein and the other through PCG1- α . Under stress conditions, SIRT1 interacts with and deacetylates FOXO1, promoting FOXO1 dependent transcription of gluconeogenic genes, thereby directing glucose metabolism from glycolysis to gluconeogenesis [84]. In addition, SIRT1 also interacts with and deacetylates PCG1- α at multiple lysine residues, thereby increasing gluconeogenic gene transcription via interaction with hepatocyte nuclear factor 4 α , with concomitant repression of glycolytic genes [255]. SIRT1 up-regulation of PCG1- α in fasted liver is correlated with increase in NAD⁺ and pyruvate, suggesting metabolic control of SIRT1 catalytic activity.

Recently, a direct regulation of insulin action by SIRT1 through its actions to control PTP1B gene expression has been demonstrated [286,323]. It was shown that SIRT1 knockdown or inhibition impairs insulin signaling and insulin-stimulated glycogen synthesis, whereas SIRT1 over-expression ameliorates existing insulin resistance and impaired glucose transport in cultured cells. Sun et al. showed that these effects are mediated in part by silencing PTP1B expression through histone H3 deacetylation. Interestingly, SIRT1 over-expression in non-insulin-resistant cells does not affect insulin signaling or glucose transport or markedly affect PTP1B expression, suggesting that SIRT1's action on insulin signaling and PTP1B is minimal in insulin-sensitive states.

1.2.2 SIRT1 and lipid metabolism

Adipose tissue plays an important role in mediating metabolic control of aging. The primary depot for fat in mammals is the white adipose tissue (WAT). It is the major site of triglyceride storage which acts as an important energy source when glucose is scarce in the blood. During fasting or starvation, triglycerides (TG) from the adipose tissue are mobilized to give free fatty acids (FFA) to be used by other tissues for energy production [80]. The impact of adipose tissue on longevity in animals was shown by adipose tissue-specific ablation of IGF receptor which resulted in about 18% longer lifespan in mice [23].

Interestingly, SIRT1 is up-regulated in CR animals, which show leanness caused by decreased insulin signaling in fat, which in turn causes decreased fat synthesis and increased mobilization of fat deposits for release into circulation. An important insulin responsive factor in WAT is the transcription factor PPAR γ . PPAR γ induces fat synthesis and WAT differentiation in response to insulin signaling. SIRT1 interacts with and represses its transcriptional activity. The nuclear receptor corepressor (NCoR) and thyroid hormone receptor (SMRT, silencing mediator of retinoid and thyroid hormone receptor) cofactors for PPAR γ . SIRT1's interaction with the PPAR γ cofactors leads to its recruitment to the PPAR γ and the adipose tissue-specific fatty acid binding protein (aP2) gene promoters. This results in PPAR γ autoregulatory synthesis and inhibition of PPAR γ mediated expression of CCAAT enhancer-binding protein α and δ , and aP2 [241]. SIRT1 over-expression and depletion experiments in mammalian cells have shown that levels of SIRT1 expression can control fat deposition. Another mechanism by which SIRT1 may play a role in lipid metabolism is believed to be through FOXO1, as SIRT1 regulates FOXO1 by deacetylation [70]. Adiponectin, an adipocyte-derived hormone whose plasma concentration

mostly correlates inversely with adiposity, is regulated by SIRT1 through FOXO1 regulation. FOXO1 forms a transcriptional complex at the mouse adiponectin promoter with C/EBP α (CCAAT/enhancer-binding protein α). SIRT1 deacetylates and enhances its interaction with C/EBP α , thereby increasing adiponectin concentrations [247].

Furthermore, the SIRT1 activator resveratrol has been shown to reduce fat accumulation and increase free fatty acid release, whereas SIRT1 inhibitor, nicotinamide (NAM) inhibits FFA release [127,241]. Consistent with this, adipose tissue from SIRT1 $^{+/-}$ mouse are less responsive to FFA release when compared to wild-type adipocytes [241]. Also, the SIRT1 $^{+/-}$ animals exhibit lower levels of blood FFA following fasting or β -adrenergic stimulation. Thus, SIRT1 inhibits insulin signaling in fat by repressing PPAR γ activity, thereby reducing adipocyte differentiation and increasing FFA release, potentially by suppressing expression of aP2.

Yet another role of SIRT1 in regulating fat metabolism was shown last year when Hallows *et al.* demonstrated that SIRT1 activates the mammalian acetyl CoA synthetase 1 (AceCs-1) via deacetylation. This is important because AceCS-1 catalyzes the synthesis of AceCoA, a key molecule in mitochondrial oxidation and lipid synthesis. SIRT1's regulation on AceCS-1 indicates that SIRT1 may modulate lipid metabolism by regulating intracellular acetyl-CoA levels [110].

1.2.3 SIRT1 and cancer

Most cancers are now recognized as an age associated disease because of its prevalence with increasing age. SIRT1 has been associated with many tumor suppressor proteins. While on one hand it deacetylates and suppresses the tumor suppressor p53 resulting in reduced p53 mediated

apoptosis in response to genotoxic stress, on the other hand, SIRT1 is required for the expression of the tumor suppressor p19(ARF), which promotes p53 stability, thereby facilitating senescence in response to chronic oxidative stress [53,198,302]. DNA damage induces SIRT1 expression, which inhibits cell death and induces stress responses by deacetylation and modulating activities of p53, forkhead box O (FOXO), E2F transcription factors and Ku autoantigen [34,55,198,216,302,305]. In theory, anti-apoptotic properties of SIRT1 could enable damaged cells to escape surveillance making them prone to cancer, thus indicating a potential role of SIRT1 in tumorigenesis. Indeed, SIRT1 was found to participate in several pathways that are involved in cancer and has been found to localize at aberrantly silenced, but not at the active promoter of tumor suppressors genes in cell lines [246]. It has been shown that the tumor suppressor HIC1 (hyper-methylated in cancer-1) directly regulates SIRT1 to modulate p53-dependent DNA-damage response [48]. HIC1, which regulates SIRT1 expression, is silenced in certain cancers resulting in up-regulation of SIRT1 and carcinogenesis. Nuclear translocation of FHL2 (four and a half LIM2), which is expressed in prostate cancer cells, correlates with the progression of prostate cancer. It has been shown that SIRT1 is also involved in mediating the anti-apoptotic effects of FHL2 through FOXO1 deacetylation, thereby promoting cancer [316]. Similarly, in hepatocellular carcinoma cells (HepG2) SIRT1 facilitates the aberrant expression of AFP (α -fetoprotein) which serves as a diagnostic marker of hepatocellular carcinoma, by deacetylating p53. It was shown that the cell-cycle and apoptosis regulator p33^{ING1b}, binds SIRT1 and inhibits its ability to deacetylate p53 to prevent carcinoma [152]. Furthermore, SIRT1 regulates the cell-cycle arrest and apoptotic activities of oncogene BCL6, which is implicated in the pathogenesis of B-cell lymphomas [18]. Consistently, SIRT1 inhibitors, cambinol and sirtinol

induces apoptosis and senescence-like growth arrest respectively, in human cancer cell lines MCF7 and H1299 [121,232].

To reconcile the discrepancy between the tendency of SIRT1 in promoting cancer and its potential to mediate CR, which reduces cancer risk, there seems to be certain compensatory SIRT1-dependent cancer-prevention pathways. For example, SIRT1 plays a pro-apoptotic role by suppressing NF- κ B activity [94,320]. It has been shown that the breast cancer associated protein, BCA3, when neddylated (modified with NEDD8) interacts with SIRT1 to suppress NF- κ B activity, thereby sensitizing breast and prostate cancer cells to TNF- α induced apoptosis [91]. Abnormal androgen receptor function has been associated with pathogenesis and progression of human prostate cancer. SIRT1 has been shown to interact with and preferentially deacetylate lys630 in the lysine motif of the androgen receptor, thereby repressing its oncogenic signaling and inhibiting prostate cancer cells from growing in response to androgen dihydrotestosterone [88].

1.2.4 SIRT1 and neuroprotection

The heart, brain and the central nervous system of mice have been identified as sites of high SIRT1 expression during embryogenesis and in adult animals [262]. In the nervous system, SIRT1 is predominantly present in the neuronal bodies [322]. SIRT1 knock-out mice have shown abnormalities in the central nervous system such as thinner retinal cell layers, indistinguishable inner and outer segments of photoreceptor cells and abnormal multiple retinal involutions [49].

The first evidence of SIRT1's role in neuroprotection came from a study on Wallerian degeneration slow mice (Wld^s) mice that were protected from axonal degeneration induced by physical or chemical injury. In this study, Milbrandt *et al.* showed that an NAD⁺ biosynthetic

enzyme, Nmnat (nicotinate mononucleotide adenylyltransferase) is primarily responsible for protection against axonal degeneration in *Wld^s* mice. Furthermore, using neuronal explants from dorsal root ganglia, they showed that protection by Nmnat is through activation of SIRT1 by increased nuclear NAD⁺ [7,16,56].

SIRT1 has also been shown to protect neurons against β -amyloid induced toxicity by inhibiting NF- κ B signaling in microglia [46]. Furthermore, SIRT1 over-expression or NAD⁺ treatment enhances α -secretase activity, which cleaves amyloid precursor protein within the A β domain and attenuates A β -peptide production. Interestingly, elevated α -secretase activity has been detected in brain specific SIRT1 transgenic mice. SIRT1 has been shown to down-regulate the serine/threonine ROCK1 (Rho kinase) expression in neurons, resulting in induction of α -secretase [248]. Since, amyloidogenic A β peptide toxicity is strongly associated with Alzheimer's disease (AD), these evidences suggest a role of SIRT1 in ameliorating AD through its neuroprotective functions. Interestingly, CR prevents AD prone mice from developing β -amyloid neuropathology, and increases SIRT1 and NAD⁺/nicotinamide ratios in the brain, suggesting that the mechanism by which CR protects neurons against β -amyloid deposition could be through modulation of SIRT1. Studies have indicated that SIRT1 may also play a protective role in Huntington's disease. It was shown that resveratrol reduces, whereas sirtinol induces death of neuronal cells in mammalian cell culture model of Huntington's disease. Also, overexpression of the worm Sir2.1 rescues neuronal dysfunction phenotype in *C. elegans* model for Huntington's disease [236].

Apart from protecting against cell death, SIRT1 can protect neurons through yet another mechanism. Increasing evidence show that maintenance of mitochondrial number and function is critical to neuronal functions, and loss of mitochondria causes many neurodegenerative diseases

[15]. PGC-1 α , one of the downstream regulators of SIRT1, acts as a master regulator of mitochondrial number and function, and is highly neuroprotective. PGC-1 α null mice show more sensitivity to neuronal loss by reactive oxygen species producing neurotoxic compounds and increasing PGC-1 α levels protects neurons against oxidative-stress-induced death [284]. Since SIRT1 is known to regulate PGC-1 α activity, it seems likely that SIRT1's neuroprotective actions are partly through regulation of PGC-1 α activity.

1.2.5 SIRT1 and other diseases

SIRT1 exhibits cardio-protective actions by protecting against cardiac hypertrophy, which is a major cause of heart failure. Heart failure is caused by inability of the heart to pump blood efficiently, which begins with cardiac hypertrophy, followed by increased apoptosis resulting in organ failure. SIRT1 has been shown to protect primary cultured myocytes from programmed cell death induced by serum starvation or activation of PARP-1, in a p53-dependent way. Using transgenic mouse with heart-specific over-expression of SIRT1, it was recently shown that mild to moderate expression of SIRT1 retards aging of the heart by inducing resistance to oxidative stress and apoptosis, whereas a high dose of SIRT1 induces cardiomyopathy [2,3,242]. Additionally, SIRT1 decetylates histone variant H2A.Z, a factor known to promote cardiac hypertrophy, thereby facilitating ubiquitin-mediated degradation of H2A.Z and protecting against heart failure [45].

SIRT1 also shows protective functions in kidney diseases. For example, SIRT1 diminishes mesangial cell apoptosis induced by oxidative stress through inhibiting p53, which prevents glomerular apoptosis in kidney diseases [170]. SIRT1 also deacetylates Smad7 resulting

in enhanced ubiquitin-dependent proteosomal degradation of Smurf1 (Smad ubiquitination regulatory factor-1), thereby protecting glomerular mesangial cells from TGF β dependent apoptotic signaling which is mediated by Smad7 [171].

SIRT1 plays a role in HIV replication by regulating Tat activity. Tat (transactivator of transcription) is a HIV-1 protein that is essential for transcriptional activation of integrated provirus. Without Tat activity, HIV-1 viral replication remains compromised because of aborted transcription due to inefficient elongation. SIRT1 deacetylates and activates the Tat protein, thereby increasing viral transcription [233].

1.3 SIRT1 ACTIVATORS

The apparent role of SIRT1 and its orthologs in extending longevity has prompted searching for compounds that can activate SIRT1 since such compounds can potentially extend lifespan in absence of CR or genetic alteration. About four years ago, the first such molecule, resveratrol, a compound produced by plants in response to stress was discovered [127]. Since then many polyphenol activators of SIRT1 have been discovered such as quercetin, fisetin, iso-liquiritigenin, piceatannol and butein. These compounds are collectively called the Sirtuin Activating Compounds or STACs.

Resveratrol, a polyphenolic compound found naturally in grape skin, and a variety of plants products such as fruits and nuts, was initially reported to increase the catalytic activity of SIRT1 *in vitro*. Subsequently, it was shown to mimic CR and increase lifespan in yeast, worms, fruit flies and fish [313]. The mechanism by which resveratrol functions is not yet clear.

However, in *S.cerevisiae*, *C. elegans* and *D. melanogaster*, the effect of resveratrol is SIRT1 dependent and is not further extended by CR, indicating that it may act through CR pathway.

Resveratrol has been shown to modulate a variety of processes in mammals in a SIRT1-dependent manner such as; neuroprotection, differentiation, tumor suppression and inflammation [5,13,173]. Consistently, resveratrol effects a number of diseases in mammals namely, cancer, heart diseases, brain damage, hearing loss, anorexia and injuries to tissues [14]. Similar to lower organism, it also elicits CR mimicking physiological changes in mammals, for example it improves tissue pathology and endurance, increases mitochondrial biogenesis, insulin sensitivity and decreases fat accumulation, blood insulin and IGF-1 [13,174]. Recently, resveratrol has been shown to increase the survival of high calorie fed mice by shifting its physiology towards standard diet mice with increased mitochondria, lower blood glucose and insulin and a hepatic gene expression profile matching the lean mice. This is consistent with the fact that increased SIRT1 activity can increase PGC-1 α activity. Also, mice fed with resveratrol show highly deacetylated PGC-1 α in multiple tissue, indicating a role of SIRT1 activity [174]. Furthermore, resveratrol induced activation of mitochondrial function has been shown to be dependent on SIRT1 mediated deacetylation of PGC-1 α . Reseveratrol has also shown other effects mimicking SIRT1 activation such as: promoting cell survival by decetylation of p53, inhibiting adipocyte differentiation by suppressing PPAR- γ , rescuing cells polyglutamine toxicity and sensitizing cells to TNF- α induced apoptosis by inducing NF- κ B deacetylation [127,236,241,320]. However, a couple of recent reports have shown that resveratrol modulates certain pathways independent of SIRT1. It was shown that resveratrol inhibits insulin response by disrupting insulin-induced IRS protein complex and this action is independent of SIRT1. In another report

resveratrol was shown to stimulate AMPK activity in neurons independent of SIRT1 and dependent on LKB1 [66,325].

1.4 SIRT1 INHIBITORS

The biological functions of SIRT1 have triggered interest in the development of SIRT1 activators and inhibitors. Silent information regulator 2 (Sir2) enzymes catalyze NAD⁺-dependent protein/histone deacetylation, where the acetyl group from the lysine epsilon-amino group is transferred to the ADP-ribose moiety of NAD⁺, producing nicotinamide and the novel metabolite O-acetyl-ADP-ribose. Soon enough it was shown that physiological concentrations of nicotinamide noncompetitively inhibit both Sir2 and SIRT1 *in vitro*. Furthermore, the degree of inhibition by nicotinamide (IC₅₀ < 50 μM) was shown to be equal to or better than the most effective known synthetic inhibitors of this class of proteins. It was proposed that nicotinamide inhibits deacetylation by binding to a conserved pocket adjacent to NAD⁺, thereby blocking NAD⁺ hydrolysis. [21]. Recently, it was demonstrated that nicotinamide inhibition is the result of nicotinamide intercepting an ADP-ribosyl-enzyme-acetyl peptide intermediate with regeneration of NAD⁺ (transglycosidation) [139].

Several chemical inhibitors of SIRT1 are also used frequently. Treatment with sirtinol, splitomicin, suramin, NF023 and NF279, at a low μM level has been shown to inhibit SIRT1 effectively. Recently, a more potent inhibitor was described, EX527 [220,243], which is approximately 1000 fold more potent than nicotinamide.

1.5 MOTIVATION AND OVERVIEW

SIRT1, the human homologue of the yeast SIR2 protein, has been shown to play a role in a wide range of cellular processes. While in lower organism such as yeast, worms and flies, increasing the copy number of SIRT1 gene extends lifespan, in mammals it shows many physiological changes that is relevant to cell survival and longevity. SIRT1 has been shown to mimic caloric restriction associated physiological changes that relates to a longer and healthier lifespan. In the past one decade, much has been discovered with regards to SIRT1's role in various cellular processes ranging from regulation of gene expression, stress resistance and endocrine signaling to metabolism. SIRT1 activators have also shown properties of promoting cell survival and delaying aging. However, a lot remains to be uncovered with regards to the mechanism or mechanisms by which SIRT1 promotes longevity in mammals. In lower organisms, such as *S. cerevisiae* and *C. elegans*, SIRT1 has been demonstrated to function through the insulin/insulin like growth factor (IGF-1) signaling pathway. However, the mechanism in mammals seems to be much more complicated with SIRT1 influencing several signaling pathways, which may be intricately regulated to impact mammalian longevity.

The goals of this study were to identify novel binding partners for SIRT1 and elucidate their potential role in SIRT1 mediated effects on mammalian cell survival and longevity. Specific aim one involved the identification of novel binding partners for SIRT1 using a yeast two-hybrid system. Following identification of TLE1 as a binding partner for SIRT1, the relevance of its association with SIRT1 was examined by analyzing its effect on SIRT1 mediated regulation of NF- κ B activity. Specific aim two involved verification of the association between

SIRT1 and eIF2 α , the second binding partner identified in the yeast two-hybrid screening. The biological relevance of this association was further investigated with regard to SIRT1 mediated cellular stress response. Specific aim three pertained to investigating the potential role of SIRT1 in the mTOR pathway, which is involved in cellular growth and proliferation, thereby playing a role in cancer.

**2.0 SIRT1 INTERACTS WITH TRANSDUCIN-LIKE ENHANCER OF SPLIT-1 TO
INHIBIT NF-KB MEDIATED TRANSCRIPTION**

Hiyaa Singhee Ghosh *et al.*

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2.1 ABSTRACT

SIRT1 is a NAD⁺ dependent deacetylase that plays a role in cellular processes such as transcriptional regulation, stress response, longevity and apoptosis. SIRT1 deacetylates histone proteins and certain transcription factors such as p53, CTIP2, FOXO and NF-κB. To identify potential SIRT1 interacting factors, we performed a yeast two-hybrid screen. The screen identified Transducin like Enhancer of split 1 (TLE1) as a possible SIRT1-interacting factor which was then confirmed by co-immunoprecipitation. TLE1 is a non-DNA binding co-repressor for several transcriptional factors including NF-κB. We demonstrate by co-transfection assays that SIRT1 and TLE1 both repress NF-κB activity. The catalytic mutant of SIRT1, SIRT1-H363Y, and the N-terminal SIRT1 fragment (1-270a.a) also show similar repression activity, suggesting that the deacetylase activity of SIRT1 may not be critical for its effect on NF-κB activity. Furthermore, analysis in SIRT1 null murine embryonic fibroblasts and HeLa cells stably expressing RNAi specific to SIRT1 or TLE1 demonstrate that both SIRT1 and TLE1 are required for negative regulation of NF-κB activity. Taken together, these results suggest that the interaction between SIRT1 and TLE1 is important for mediating repression of NF-κB activity.

2.2 INTRODUCTION

SIRT1, a NAD⁺-dependent deacetylase, is the human homolog of the yeast Sir2 protein which plays a role in transcriptional regulation, cell cycle, differentiation, metabolism, DNA damage response, apoptosis and longevity. SIRT1 is classified as a class III histone deacetylase (HDAC-III), because of its dependence on NAD⁺ as a cofactor for its activity, as oppose to the class I and II histone deacetylase that use zinc as a cofactor [122]. Accordingly, the SIRT1 deacetylase is not inhibited by the general histone deacetylase inhibitor trichostatin A, and instead have its own specific inhibitors such as nicotinamide [69,181]. Other than deacetylating histone proteins, such as H1, H3 and H4, SIRT1 also deacetylates a number of transcription factors and other proteins. SIRT1 interacts with the bHLH repressor proteins, HES1, HEY2 [288] and the COUP-TF interacting protein 2 (CTIP2) [270], to confer transcriptional repression. The other non-histone substrates of SIRT1 include p53, PCAF, MyoD, p300, Ku70, PPAR γ and PGC1 α [28,34,89,218,241,255]. SIRT1 is believed to play a protective role under caused genotoxic or oxidative stress conditions. SIRT1 regulates the Forkhead transcription factors, Foxo1, 3 and 4 to regulate cellular resistance to oxidative stress [34,63,216,298,316]. SIRT1 also increases cell survival to DNA damage and oxidative stress by inhibiting p53-mediated apoptosis [302]. However, the role of SIRT1 deacetylase activity in regulating cellular stress response has been controversial with reports that demonstrate that overexpression of SIRT1 or inhibition of its catalytic activity didn't affect cell viability in response to stress [282,302].

Nuclear factor κ B (NF- κ B) is a transcription factor that regulates the expression of a over 150 target genes majority of which participate in host-immune response and stress response pathways [234]. Apart from immune induction of NF- κ B, the various physiological stress conditions that induce NF- κ B activity are ischemia/reperfusion, liver regeneration and

hemorrhagic shock. Physical stress in the form of irradiation and oxidative stress to cells or ER stress also induce NF- κ B activity. NF- κ B is also activated both by environmental stresses, such as heavy metals or cigarette smoke, and by therapeutic drugs, including various chemotherapeutic agents. Accordingly, a large variety of stress response genes, such as the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are in turn activated by NF- κ B. Because of its role mediating immune response and cellular stress response, NF- κ B is believed to relay the information of an imminent stress and at the same time enact a response by promoting the transcription of genes whose products alleviate the stress condition.

The vertebrate Rel/NF- κ B family is comprised of five proteins, c-Rel, RelA, RelB, p105/NF- κ B and p100/NF- κ B, all of which are structurally related. To regulate the expression of its target genes, these dimers bind to a set of related 10 bp DNA sites, collectively called κ B sites. The most common transcriptionally active NF- κ B in mammals consists of a heterodimeric protein complex p50-RelA (p65) which is specifically called NF- κ B. In most cells, Rel/ NF- κ B transcription complexes are present in a latent, inactive state in the cytoplasm where they are bound to an inhibitor (I κ B). One of the major step regulating NF- κ B activity is removal of I κ B from NF- κ B:I κ B complexes, a processes that is regulated by controlling I κ B degradation via IKK-mediated phosphorylation. Various stimuli, such as mitogens, growth factors and cytokines can rapidly activate these transcription complexes by freeing them from their inhibitor and enabling them to translocate to the nucleus to regulate transcription. Posttranscriptional modification of p65 is also considered critical for NF- κ B activity. A number of kinases, such as protein kinase A catalytic subunit (PKAc), casein kinase II (CKII), PKC ζ , and IKK have been shown to be directly associated with the cytosolic NF- κ B:I κ B complex, and implicated in p65 phosphorylation [95]. Furthermore, regulation of acetylation on p65 has been shown to be

critical for NF- κ B activity, which further emphasizes the role of p65 phosphorylation in regulating NF- κ B activity, as histone acetylase (HAT) recruitment to p65 is dependent on its phosphorylation [95]. It has been proposed that acetylation of p65 determines the efficiency of its interaction with I κ B α . Therefore, the acetylated p65 that enters the nucleus is refractory to inhibition by I κ B α , but following HDAC3 recruitment, the deacetylated p65 associates with I κ B α and is exported to the cytoplasm, downregulating NF- κ B activity. Accordingly, members of the histone deacetylases, HDAC1, HDAC2 and HDAC3, have been shown to regulate the activity of NF- κ B by deacetylating RelA/p65, increasing its association with I κ B and reducing transcriptional activity. Acetylation of p65 at lysine residues 122 and 123 decreases DNA binding affinity of the heterodimer, whereas acetylation at lysine residues 310 and 221 increases DNA binding and impairs its association with I κ B [47]. Notably, SIRT1 has been reported to repress NF- κ B mediated gene expression by deacetylating the p65 subunit of NF- κ B [320].

NF- κ B activity is also regulated by members of transducin-like Enhancer of split1 (TLE1), a family of transcriptional repressors that are mammalian homologs of the *Drosophila* Groucho protein family. The Groucho family includes three types of proteins. The larger proteins such as Groucho and its mammalian homologs, transducin-like enhancer of split (TLE) 1 through 3, share five domain structures. These proteins exhibit a common feature including an amino-terminal glutamine-rich region (Q domain), a glycine/proline-rich region (GP domain), a CcN domain containing a casein kinase II site and nuclear localization sequence, a serine/proline-rich region (SP domain), and COOH-terminal WD40 repeats [78,285]. Three of these domains the Q, CcN, WD40 domains are most highly conserved. A shorter protein, the human TLE4, contains all the domains except for the amino-terminal Q domain. Shortest proteins in the Groucho family, which contain only the Q domain and the GP domain, are

designated as AES or the Groucho-related gene (Grg) [201,212]. Significant homology is observed in the Q domain between AES and other Groucho proteins except for TLE4. *Drosophila* Groucho protein and its mammalian homologs, TLEs, have been shown to serve as non-DNA binding corepressors for several transcription factors including the Hairy-related proteins, Runt domain proteins, Engrailed, Dorsal, and lymphoid enhancer factor-1/T-cell factor. The WD40 repeats in Groucho and TLEs appear to mediate protein-protein interaction with relevant DNA-binding proteins, such as Engrailed and Hairy [38,71,147,237,295].

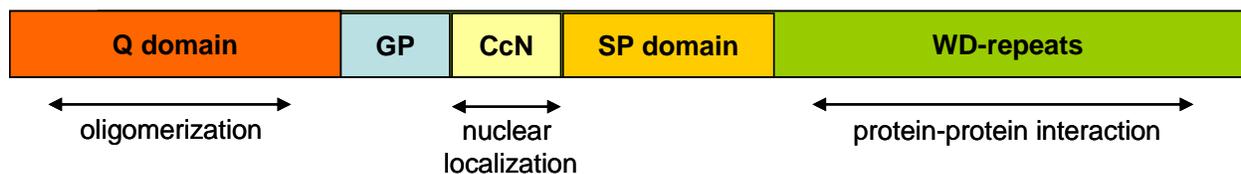


Figure 2.1 Structure/function relationships in the Gro/TLE family.

Functional domains of Gro/TLE corepressors. The conserved Q and WD-repeat domains are in color. The open boxes represent the more flexible central regions in Gro/TLE proteins. Two separate regions, the GP and the SP domains, are known to possess repression activity (marked by I and II). A pair of putative amphipathic α -helical motifs are indicated by two lines at the top of the Q domain and are labeled AH1 and AH2.

Based on the findings that the active form of Gro is a tetramer and that Gro makes specific contacts with the histone deacetylase Rpd3 as well as histones, a model has been proposed for Gro-mediated transcriptional repression where in: after recruitment to the template via a direct interaction with sequence specific DNA-binding transcription factors, the ability of Gro to oligomerize together with the favorable interactions between Gro and histones results in the nucleation of a Gro polymer that may spread along the DNA template. The template-bound

Gro-polymer then provides a high-affinity interface for the recruitment of key chromatin-modifying factors, including the histone deacetylase Rpd3. These components may subsequently serve to establish and/or maintain a transcriptionally silenced chromatin structure.

Several lines of evidence suggest that Gro-homologs in mammals may perform functions in development and cell-fate determination that are homologous to those carried out by their *Drosophila* counterparts. For example, the expression patterns of individual Gro/TLE family proteins during mouse embryonic development are consistent with roles in segmentation, central and peripheral neurogenesis, as well as epithelial differentiation [67,167,184]. In addition, TLE proteins are temporally and spatially coexpressed with *Notch* as well as other members of the Notch pathway consistent with possible roles in the multiple cell fate decisions governed by this pathway [102]. Mammalian Gro/TLE proteins are also known to interact directly with Hairy-related bHLH transcription factors in mammals — the so called HES (Hairy/Enhancer of split) family of proteins [78,79,101]. The conserved carboxyl-terminal WRPW motifs in these HES proteins are sufficient for both the recruitment of Gro/TLE corepressors and transcriptional repression [79,101]. In addition, direct and functional associations have also been established between the TLE corepressors and the mammalian counterparts of the *Drosophila* Tcf and Runt proteins [9,133,185]. The *Drosophila* Groucho protein directly binds the *Drosophila* homolog of NF- κ B, Dorsal, and converts it from a transcriptional activator to a repressor [71]. Similar to this, the mammalian Groucho homologs, TLE1 and the Amino-terminal Enhancer of Split (AES) have also been shown to physically interact with the p65 subunit of NF- κ B to inhibit its activity through an unknown mechanism [293].

In our study, we performed a yeast two-hybrid analysis to screen the human spleen cDNA library for identifying potential novel binding partner for SIRT1 to elucidate the

underlying mechanism of SIRT1 in mediating cellular stress response and longevity. Our analysis identified the Transducin like Enhancer of Split-1 (TLE1), as potential novel binding partner for SIRT1 which was further confirmed in mammalian cells by co-immunoprecipitation. As mentioned above, both SIRT1 and TLE1 play a role in regulating NF- κ B activity. While SIRT1 was suggested to deacetylate p65 to inhibit NF- κ B activity, the mechanism by which TLE1 repressed NF- κ B activity was not elucidated. Hence, we investigated the association of SIRT1 and TLE1 with regards to their role in regulating NF- κ B activity. We demonstrate here, that repression of NF- κ B activity mediated by SIRT1 and TLE1 is enhanced when both the proteins are overexpressed together. More significantly, neither SIRT1 nor TLE1 was able to repress NF- κ B activity in the absence of the other. Interestingly, our results also suggest that the catalytic function of SIRT1 is not required for the repression of NF- κ B activity. Thus, we propose that the association between SIRT1 and TLE1 plays a critical role in mediating SIRT1 and TLE1 mediated repression of NF- κ B activity and possibly the activity of other transcription factors, potentially through a deacetylase independent mechanism.

2.3 MATERIALS AND METHODS

2.3.1 Cell culture

HeLa cells were purchased from American tissue culture consortium (ATCC) and cultured in DMEM media (Dulbecco's modified Eagle's media, Cellgro # 10-013-CV) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100U penicillin, 100U streptomycin and 0.25µg/ml amphotericin B (antibiotic-antimycotic; GIBCO # 15240-062), Hepes buffer and 2mM L-glutamine. Cells were cultured at 37⁰C, 19% O₂ and 5% CO₂. The SIRT1-wild-type and SIRT1 null mouse embryonic fibroblasts were a gift from Dr. Michael McBurney, University of Ottawa, Canada. These cells were also maintained in the DMEM growth media. SIRT1 MEFs were cultured at 37⁰C, 15% O₂ and 5% CO₂. Cells were harvested by treating cells with trypsin-EDTA (Cellgro, # 25-053-C1) for 2mins in 37⁰C incubator. Cells were washed by re-suspending cells in growth media and centrifugation at 5000g in a table-top centrifuge.

2.3.2 Construction of plasmids for yeast two-hybrid screen

A point mutation was carried out using QuikChange kit (Stratagene) to create an in frame BamHI-SalI cloning site in the yeast expression vector pGBKT7 (Clontech Laboratories) using the primers: 5'-GGA GGC CGA ATT CCC GGG ATC CGT CGA CCT GCA G-3' and 5'-CTG CAG GTC GAC GGA TCC CGG GAA TTC GGC CTC C-3'. 12 cycles of PCR using *PfuTurbo* DNA polymerase (Stratagene) was performed as per manufacturer's protocol. The PCR product was digested with the *Dpn I* restriction enzyme to eliminate the parental supercoiled, methylated dsDNA. The PCR synthesized plasmids were then transformed into XL-Blue supercompetent

cells and plasmid was purified using Maxiprep Kit (QIAGEN). The full length SIRT1 cDNA was obtained from pYESir2-puro, a generous gift from Robert Weinberg [302], and ligated in frame into the BamH1/Sal1 Gal4-DNA-binding domain of the frame-shifted yeast expression vector to generate Gal4DBD- SIRT1 (pGBKT7- SIRT1) for the yeast two-hybrid screen.

2.3.3 Yeast two-hybrid analysis

Yeast two-hybrid screening was performed according to the Clontech Matchmaker protocol using the full-length SIRT1 as bait for the human spleen cDNA library (Clontech Laboratories). Briefly, the cDNA library was first titered and then amplified to isolate 10.5×10^6 (3 times the size of the library) independent colonies. The library was plated on LB + 100 μ g/ml ampicillin plates at a high enough density so as to get approximately 40,000 colonies per plate (fully confluent 150 mm plates) using 5 mm diameter sterile, glass beads. Plates were incubated at 37°C for 18-20 hrs. 5ml of sterile LB + glycerol was added to each plate and the colonies were scraped off using a glass spreader. The pooled colonies were incubated at 30°C for 3 h with shaking at 200 rpm. Library plasmid was extracted using Megaprep Kit (QIAGEN).

AH109 yeast cells were transformed with pGBKT7- SIRT1 using the PLATE (PEG/LiAc/Tris-EDTA) transformation protocol (Clontech Matchmaker). For verification of fusion protein expression, transformants were selected on SD-trp plates. Protein extraction from yeast was performed using SDS/Urea method (Yeast protocol handbook, Clontech). Western blot analysis was performed on the extracted protein using monoclonal anti- SIRT1 antibody (Upstate). The large scale, simultaneous co-transformation protocol was performed according to manufacturer's instructions (Clontech). 50 μ g of the human cDNA expression library fused to Gal4-transactivation domain in the pACT2 vector (Clontech) (AD/library) and 100 μ g of the full

length SIRT1 fused to the Gal4 DNA-binding domain in the pGBKT7 yeast expression vector (DNA-BD/bait) was used. Co-transformants were selected on high-stringency SD/-H/-A/-L/-T/+ 2.5mM aminotriazol plates at 30°C. Plasmids expressing Gal4-DBD-p53 and Gal4-AD-T antigen were used as positive control whereas Lamin C and T-antigen were used as negative control. The 78 colonies that grew on high stringency selection plates were streaked on SD/-L/-T/+X-β-gal plates for β-galactosidase assay for further confirmation of positive clones. Yeast plasmids were rescued from clones that were positive for the β-galactosidase activity using the Hoffman and Winston method (Clonetech Laboratories) and analyzed by nucleotide sequencing. The cDNA sequences were compared with NCBI GenBank database for identification of the interacting proteins.

2.3.4 Design of small interfering RNA targeting vectors

Already published RNAi sequence for knocking down SIRT1 and TLE1 [148,192] was used to design sequence of two complementary oligonucleotides suitable for cloning into the pSIREN RetroQ siRNA expression system, according to the instructions in the BD Knockout RNAi Systems User Manual (BD Biosciences, PT3739-1). The synthetic oligonucleotide sequences designed against SIRT1 and TLE1 were ordered from ‘Operon Technologies, Inc’, Alameda, CA. Complementary sequences used for knocking-down SIRT1 is :

5' GATCC**GCTGGAGCTGGGGTGT**

CTGTTTCAAGAGAAACAGACACCCAGCTCCAGCTTTTTTCTAGAG3' and 5'

G**CGACCTCGACCCACAGACA**AAGTTCTCT**TGTCTGTGGGGTCGAGGTC**GAAAAAAGATCTCT

TAA 3' and for knocking-down TLE1 is: :

5' GATCCG**CGTGCCAAACAGGTGACCA**TTCAAGAGAT**TGGTCACCTGTTTGGCACG**CTTTTTTTC
TAGAG3' and

5' AATTCCTCTAGAAAAAG**CGTGCCAAACAGGTGACCA**TCTCTTGAA**TGGTCACCTGTTTGGC**

ACGCG 3' [94,148,192]. The oligonucleotides were annealed and ligated into RNAi-ready pSIREN RetroQ vector according to instructions in the BD Knockout RNAi Systems User Manual (BD Biosciences, PT3739-1). Ligation products were transformed into Fusion-blue competent cells and grown on agar plates containing 100 µg /ml ampicillin (Sigma Aldrich, St Louis, MO) and incubated overnight at 37°C. Single bacterial colonies were grown in 5 ml LB media supplemented with 100 µg/ml ampicillin and plasmid DNA was extracted using the QiaPrep Spin Miniprep Kit (Qiagen, Valencia, CA). Restriction digest analysis with restriction site (BamHI/EcoRI) within the shRNA oligonucleotide sequence was used to identify bacterial cultures with a properly cloned plasmid. These bacterial cultures were inoculated in 500 ml of LB and grown overnight at 37°C; plasmid was extracted from these cultures using a Qiagen Maxiprep Kit. The pSIREN RetroQ expression vectors contain the Human U6 RNA polymerase III promoter and it also expresses a puromycin-resistance gene enabling selection of infected cells. Each siRNA consists of a 19-base-pair sequence corresponding to the sense strand of the targeted RNA and a 19-base-pair anti-sense strand, separated by a short 6-base-pair sequence. A negative control vector (luciferase shRNA Negative Control Vector) expressing a short RNA sequence that does not form a hairpin, was used as a control for retroviral infection and transcription from the U6 promoter (Clontech).

2.3.5 Production of retrovirus

3×10^6 HEK293T cells were plated in 10cc culture dishes. Cells were incubated at 37°C in 10 ml of DMEM-10% FCS with 2 mM L-Glutamine, without antibiotics. At 70-80% confluency, cells were transfected with 10 µg retroviral plasmid (pSIREN RetroQ-SIRT1-RNAi or pSIREN RetroQ negative control vector) and 5µg amphotropic envelope packaging plasmid using LipofectamineTM2000 transfection reagent (# P/N 52887, Invitrogen, Grand Island, NY), as per manufacturer's protocol. After 6 hours, the transfection media on the cells was replaced with antibiotic-antimycotic supplemented media. After 48 hours post-transfection, media was collected from the cell cultures and spun at 3000g in a table-top centrifuge. The supernatant was used for retroviral infection of HeLa cell cultures. Life of virus is about 6-8 hrs at 37°C, so if there was extra virus, it was stored on ice if used within a couple of hrs of harvesting or stored at -80°C, good to use within 6 months.

2.3.6 Retroviral infection and selection of stable cell lines.

2×10^6 HeLa cells/ retroviral infection and one mock infection were plated in 10cc culture dishes in growth media. 6ml of retroviral supernatant were mixed with 2ml growth media and 4µg/ml Polybrene (Sigma Aldrich, St Louis, MO) and added in HeLa cell culture dishes. Cells were incubated for 48hrs at 37°C. The infection media was replaced with fresh growth media supplemented with 1µg/ml puromycin (SIGMA # P7255) and cells were selected for 7 days. Medium was changed after 2 days when all of the cells from the mock-infected dish died. Newly generated cell lines stably expressing shRNAi for SIRT1 were grown in normal growth media

and examined by western blot analysis for knock-down of SIRT1 protein using anti-SIRT1 antibody (Upstate # 05-707).

2.3.1 Transfection and Luciferase assay

HeLa cells were seeded in a 24 well plate at ~ 80% confluency in DMEM growth media without antibiotic-antimycotic and cultured for overnight. Next day, cells were transfected with 50ng NF- κ B luciferase reporter plasmid (Stratagene) along with 50ng renilla-luciferase reporter, pRL-TK plasmid (Promega), using Lipofectamine2000 transfection reagent (Invitrogen) according to manufacturer's protocol. At 24 hrs post-transfection, the cells were treated with 10ng/ml TNF- α (R&D System) for 3 hrs. Cells were harvested followed by protein extraction using NP40 lysis buffer. Luciferase assays were performed using the Luciferase Assay System (Promega). Renilla luciferase activity was used as an internal control for transfection efficiency. The data was presented as percentage luciferase activity (mean \pm S.E) of three independent transfections relative to no-treatment.

2.3.2 Whole cell lysate preparation and protein estimation

Whole cell lysates were made by lysing cells in NP-40 lysis buffer (20mM TrisHCL, pH8.0, 137mM NaCl, 10% glycerol, 1% nonidet P-40, 2mM EDTA). 1X protease inhibitor cocktail (SIGMA # P2714) was added to lysis buffer just before lysis. Cells were lysed for 20 mins on ice and lysate was separated from cell debris by centrifugation for 20 mins at 13000g in a micro-centrifuge at 4°C. Protein concentration of the cell lysates were estimated by Bradford assay. 2 μ l

of lysate was added to 1ml BioRad protein assay reagent (#500-0006, BioRad Labs, Hercules, CA). 0, 1, 2, 3, 4, 6 and 8 μ g BSA (SIGMA # A2153) from 1mg/ml stock was used to plot the standard curve. Readings were taken at 595nm wavelength.

2.3.3 Co-immunoprecipitation assay

HeLa cells were transfected with plasmids expressing myc-tagged TLE1 and flag-tagged SIRT1. Whole cell protein was extracted using NP40 lysis buffer followed by protein quantification using the Bradford method as described above. Whole cell extract containing 500 μ g protein was incubated with 1 μ g of anti-flag mouse monoclonal antibody (Chemicon # MAB3118) or anti-myc mouse monoclonal antibody, clone 9E10 (Upstate # 05-419) for overnight at 4°C on a rotator. Mouse normal IgG (Santa Cruz Biotech # sc-2025) was used as negative control. 30 μ l of a 50% Protein G-sepharose bead slurry (SIGMA # P4691) was added and incubated for 1.5 hrs at 4°C. The beads were then washed five times in 1ml cold lysis buffer. Centrifugation during washes was carried at 2000g. After the final wash, the supernatant was carefully discarded by suction using a fine tip pipette. The beads were then resuspended in 25 μ l of 2X-Lamlli sample loading buffer (BioRad # 161-0737) and boiled for 5 mins to elute the antibody bound complexes. Proteins were resolved by SDS-PAGE and analyzed by western blotting.

For endogenous protein-protein interaction studies, whole cell extract from HeLa cells was used for coimmunoprecipitation as described above using anti-TLE1 polyclonal antibody (SantaCruz Biotech # sc-9121) or anti-SIRT1 monoclonal antibody (Upstate # 05-707) for immunoprecipitation and anti-SIRT1 monoclonal antibody or anti-TLE1 polyclonal antibody respectively for Western blot analysis. Rabbit normal IgG (# sc-2027) and Mouse normal IgG (# sc-2025) was used appropriately as negative control.

2.3.4 Western blotting

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the experiment, immunoprecipitated samples or 50 μ g lysate protein was loaded in 12% polyacrylamide gels. Separating gels were made using Protogel 30% w/v acrylamide: 0.8% w/v bis-acrylamide (# EC-890, National Diagnostic), 1.5M Tris-HCL pH8.8, 10% ammonium persulfate (APS), 10% SDS and 0.1% TEMED (BioRad # 161-0801). 4% Stacking gel was made using same above reagents, except 1.5M Tris-HCL pH6.8 instead of pH 8.8. Gels were run at 10mA for one minigel or at 50V in 1X-SDS-running buffer. Precision plus dual color protein marker (BioRad # 161-0374) was used as molecular weight standard. Samples resolved on SDS-PAGE were transferred on PVDF membrane (Immobilon-P, Millipore, Bedford, MA) in a semidry transfer machine (Bio-Rad, Richmond, CA) at 15V for 1 hr. Membranes were then incubated in blocking buffer (1X PBS, 5% w/v non-fat dry milk, 1% Tween-20) for 1hr at RT on a rocker. After blocking, membranes were washed three times with wash buffer (1X PBS, 1%Tween-20) for 5mins each. The washed membranes were then incubated with primary antibody for 2hrs at RT on a rocker. Primary antibody dilutions used were as follows: 1:1000 anti-TLE1 and 1:2000 for anti-SIRT1, anti-flag-tag and anti-myc-tag antibody. Following primary antibody staining, membranes were given three washes with wash buffer (PBST), 10 min each and incubated with corresponding HRP-conjugated-secondary antibody (1:5000) for 1 hr at RT on a rocker. Membranes were then washed three times in PBST and proteins were visualized by enhanced chemiluminescence (# NEL103, PerkinElmer Life Science, Inc).

2.4 RESULTS

2.4.1 SIRT1 interacts with TLE1 *in vivo*

To identify potential binding partners of SIRT1, we performed a yeast two-hybrid analysis to screen human spleen cDNA library using SIRT1 fused to the Gal4-DNA binding domain (DBD) as the bait.

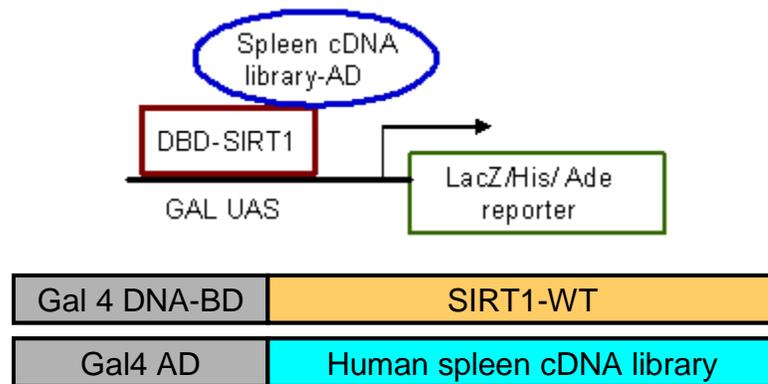


Figure 2.2 Schematic of the Yeast-two hybrid assay, and the bait and library protein constructs

Schematic showing the interacting fusion proteins: GAL4 DBD-SIRT1 and AD-human spleen cDNA library, at the GAL4 upstream activating sequence in a yeast two-hybrid assay. Interaction between the bait protein and the library protein leads to transactivation of the GAL4 promoter resulting in activation of the LacZ/ His/ Ade reporter. SIRT1 full length cDNA fused with the Gal4 DNA Binding Domain (DBD) in the pGBKT7 framshifted vector was used as the bait protein. Human spleen cDNA library clones fused with the Gal4-transactivation domain as the prey Activation Domain (AD) proteins.

We screened approximately 10.5×10^6 transformants of a human spleen cDNA library fused to the GAL4 activation domain (AD) in the AH109 yeast strain. A high stringency nutritional selection media (SD-H/-A/-L/-T+2.5mM AT) was used for the primary selection of positive clones. The 78 colonies that grew on high stringency selection plates were streaked on

SD/-L/-T/+X-β-gal plate for β-galactosidase assay for further confirmation of positive clones. As shown in Figure 2.4 clones showing blue color were finally selected as positive clones.

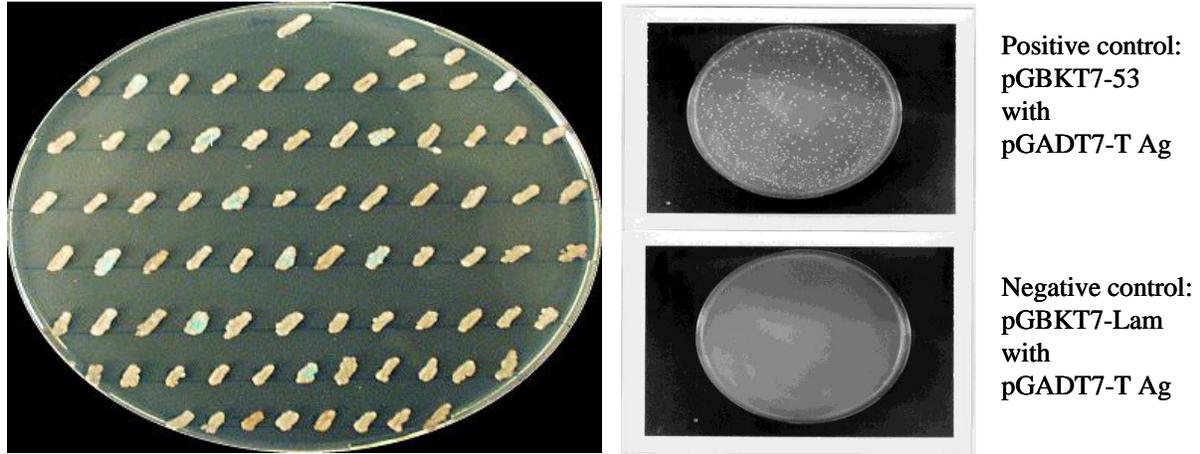


Figure 2.3 β-galactosidase assay for verification of positive clones

Comparison of the cDNA sequences from the positive clones on β-galactosidase assay with NCBI GenBank database identified a number of interesting potential binding partners for SIRT1. As shown in table 2.1, we identified two positive clones identifying TLE1, and one clone each for eIF2α, carnitine palmitoyl transferase and metallothioneine IIA.

Table 2.1 Protein identified as potential binding partners for SIRT1 in the Yeast two-hybrid screen

Clone number	Protein	Protein function
55, 60	Transducin Like-Enhancer of Split-1 (TLE1)	Transcriptional corepressor Required for developmental processes
23	Eukaryotic initiation factor2- alpha	Required for stress induced translation

	(eIF2 α)	attenuation
28	Carnitine palmitoyl transferase	Rate controlling enzyme in beta-oxidation
69	Metallothionine-IIA	Prevents cells from apoptosis induced by oxidative stress and metals.

The identification of TLE1 as a potential binding partner of SIRT1 in the yeast two-hybrid screen was of particular interest because of a number of reasons: a) SIRT1 was known to play a role in gene silencing by histone deacetylation and regulation of other transcription factors, whereas TLE1 was a transcriptional corepressor that was required by various transcription factors for repressing gene expression, b) TLE1 was reported to negatively regulate NF- κ B mediated transcription through an unclear mechanism and c) data from our laboratory suggested that SIRT1 represses NF- κ B activity, which was later published by Mayo et al, demonstrating that SIRT1 deacetylates the p65 subunit to inhibit NF- κ B [320].

To further confirm the interaction between SIRT1 and TLE1, we reintroduced the plasmids expressing the GAL4-AD-TLE1 fusion rescued from two positive yeast two-hybrid clones into Y187 yeast strain along with plasmid expressing Gal4-DBD-SIRT1. Protein-protein interaction was demonstrated by the development of blue coloration on X- β -gal plates. As shown in Figure 2.5, blue colonies were observed only with clones expressing SIRT1-DBD and TLE1-AD and the positive control consisting of plasmids expressing DBD-p53 and AD-T antigen, but not with the controls consisting of plasmids expressing SIRT1 and AD, DBD and TLE1 or DBD-Laminin C and AD-antigen.

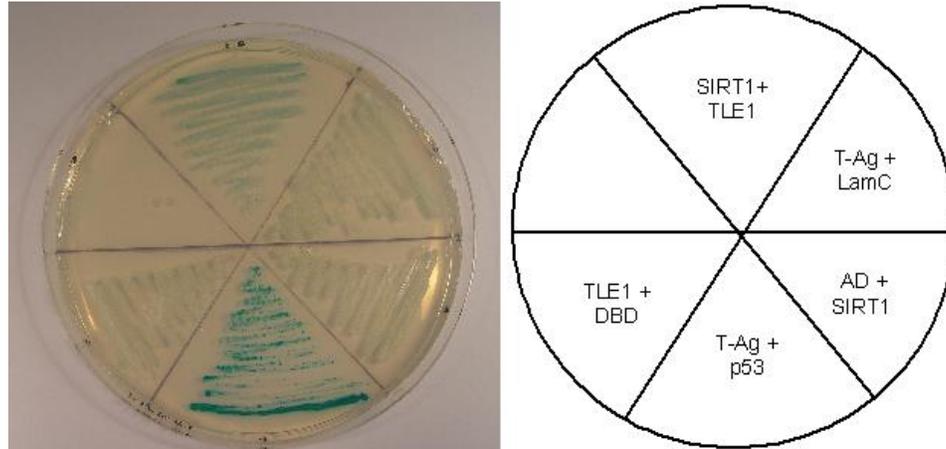


Figure 2.4 Verification of SIRT1-TLE1 interaction by β -galactosidase assay

Yeast strain Y187 was transformed with DBD-SIRT1 and AD-TLE1 plasmids rescued from positive clone in first β -Galactosidase assay. Blue coloration on SD/-L/-T/+X- β -gal plates is only seen in clones with SIRT1 and TLE1 or T-Ag and p53 (positive control). Other control sets are as follows: plasmids expressing AD-TLE1 and DBD, DBD-SIRT1 and AD, DBD-T-antigen and AD-p53, DBD-T-antigen and AD-Lamin C.

To demonstrate that SIRT1 interacts with TLE1 in mammalian cells, the ability to co-immunoprecipitate endogenous SIRT1 and TLE1 as well as transiently expressed, epitope-tagged versions of the proteins was examined. As shown in figure 2.6 (A) immunoprecipitation of whole cell extracts from HeLa cells with anti-TLE1 or anti-SIRT1 antibodies followed by Western blot analysis with anti-SIRT1 or anti-TLE1 antibody respectively, demonstrated that the endogenous SIRT1 and TLE1 proteins interact under physiological conditions. Consistent with this observation, FLAG-tagged SIRT1 and Myc-tagged TLE1 also were co-immunoprecipitated from transfected HeLa cells (B).

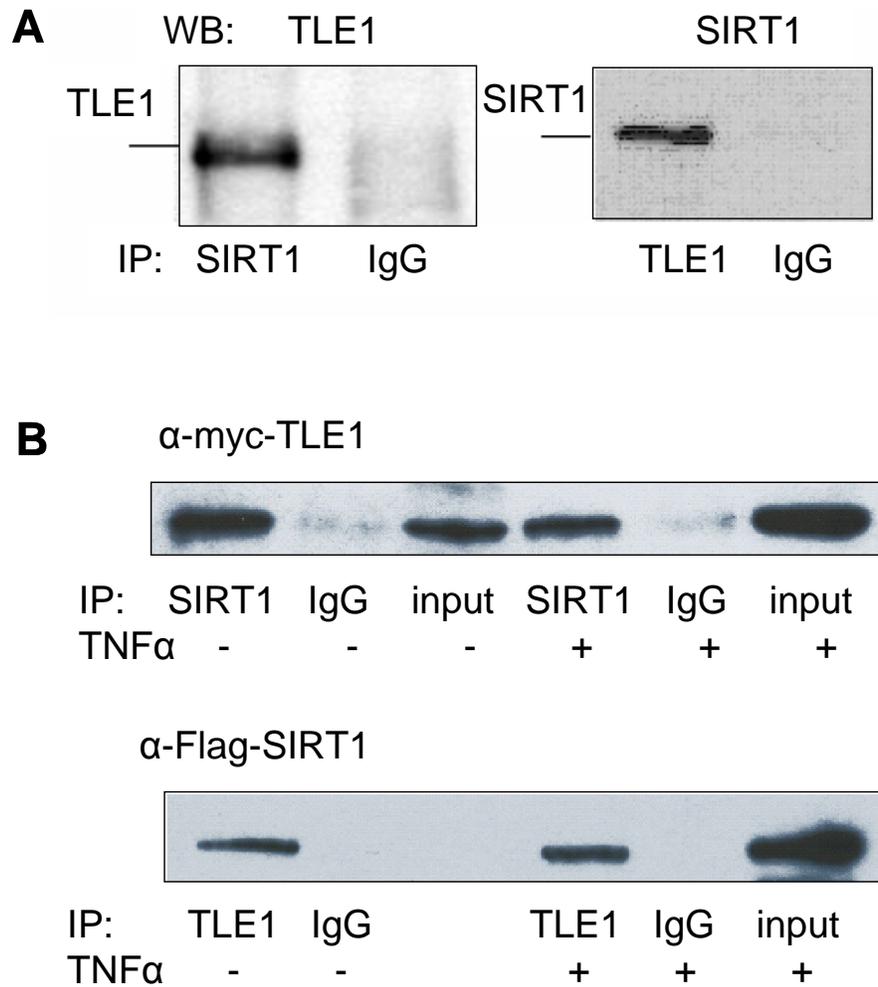


Figure 2.5 Co-immunoprecipitation of SIRT1 and TLE1

SIRT1 and TLE1 interact in mammalian cells: **A**, Whole cell extracts from HeLa cells were immunoprecipitated with anti-SIRT1 and anti-TLE1 antibody. For control, the extracts were immunoprecipitated with mouse monoclonal IgG and rabbit polyclonal IgG respectively. The immunoprecipitated proteins were immunoblotted with anti-TLE1 and anti-SIRT1 respectively. **B**, HeLa cells were transfected with Myc-TLE1 and Flag-SIRT1 and whole cell extracts were prepared 24 hrs post-transfection with or without TNF α induction. SIRT1 and TLE1 were immunoprecipitated using anti-SIRT1 and anti-TLE1 antibody respectively, followed by Western blotting using anti-Myc and anti-Flag respectively.

2.4.2 SIRT1 and TLE1 repress NF- κ B mediated transcription

Both SIRT1 and TLE1 have been shown to regulate transcription mediated by NF- κ B [293,320]. To examine the effect of SIRT1 and TLE1 on NF- κ B mediated gene expression, a co-transfection assay was performed using a luciferase reporter plasmid (5x- κ B-TATA-Luc) containing five tandem repeats of the NF- κ B DNA binding sequence upstream of a TATA box. As shown in Figure 2.7, overexpression of SIRT1 and TLE1 both inhibited NF- κ B mediated luciferase reporter expression in HeLa cells and the inhibitory effect was slightly enhanced when Sirt1 and TLE1 were both overexpressed.

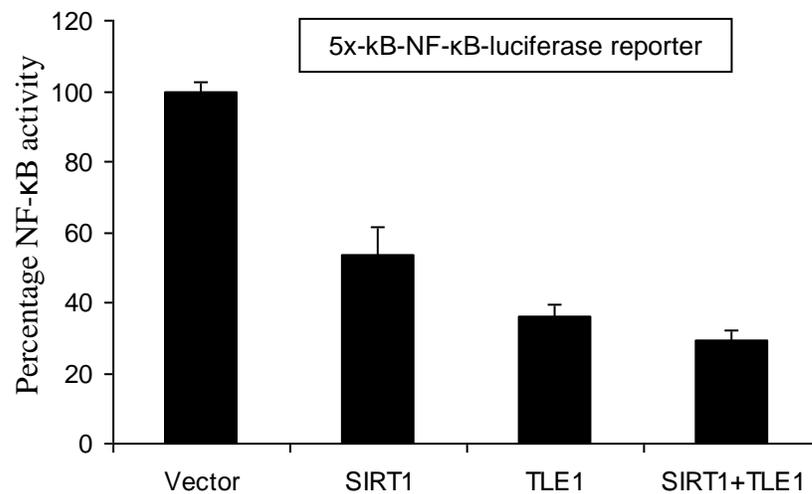


Figure 2.6 SIRT1 and TLE1 repress NF- κ B dependent luciferase gene expression in HeLa cells

The data is presented as fold decrease in luciferase activities. HeLa cells were transfected with 50ng of 5x- κ B-TATA-Luc reporter plasmid and 500ng of the indicated expression plasmids. pCMV-3B, the vector control, was included such that all transfections had equivalent amounts of expression plasmids. Renilla luciferase was used as the internal transfection control. 24 hr post-transfection, cells were induced with TNF α for 3hr and harvested for luciferase analysis.

To investigate further the ability of the SIRT1 and TLE1 interaction to inhibit NF- κ B mediated transcription, a dose response analysis was performed using the NF- κ B dependent luciferase reporter (Figure 2.8). At lower doses (50 ng and 150 ng) of SIRT1 and TLE1 expression plasmids, repression of NF- κ B activity was increased by about 30-40% by co-expression compared to the repression mediated by SIRT1 and TLE1 individually. However, at the higher dose (500ng) of expression plasmids, the difference between level of repression mediated by SIRT1 and TLE1 individually compared to when both the proteins were overexpressed was reduced. This result suggests that co-expression of SIRT1 and TLE1 together, at least at lower levels of expression, can mediate repression of NF- κ B activity more efficiently than either factor alone.

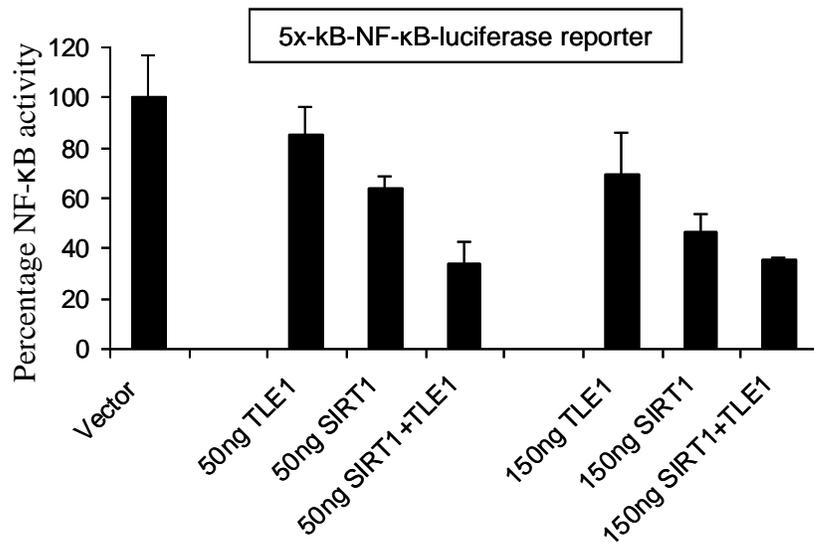


Figure 2.7 SIRT1-TLE1 co-expression enhances NF- κ B repression by SIRT1 or TLE1 individually

The data is presented as fold repression of luciferase activity. Cells were transfected with 50ng of 5x- κ B-TATA-Luc reporter plasmid and 0, 50 and 150ng of the indicated expression plasmids. pCMV-3B, the vector control was included such that all transfections had equivalent amounts of expression plasmids. Renilla was used as the internal transfection control. 24 hr post-transfection cells were induced with TNF α for 3hr and harvested for luciferase assay.

2.4.3 Deacetylase activity of SIRT1 is not required for suppression of NF- κ B activity

It has been reported that SIRT1 represses NF- κ B activity by decetylating its p65 subunit, reducing its ability to transactivate [320]. Interestingly, the catalytic mutant of SIRT1, SIRT1-H363Y was able to repress NF- κ B activity in a co-transfection assay, similarly to wild-type SIRT1 (Figure 2.9). In addition, the inhibitory effect of the catalytic mutant was enhanced by co-transfection with TLE1.

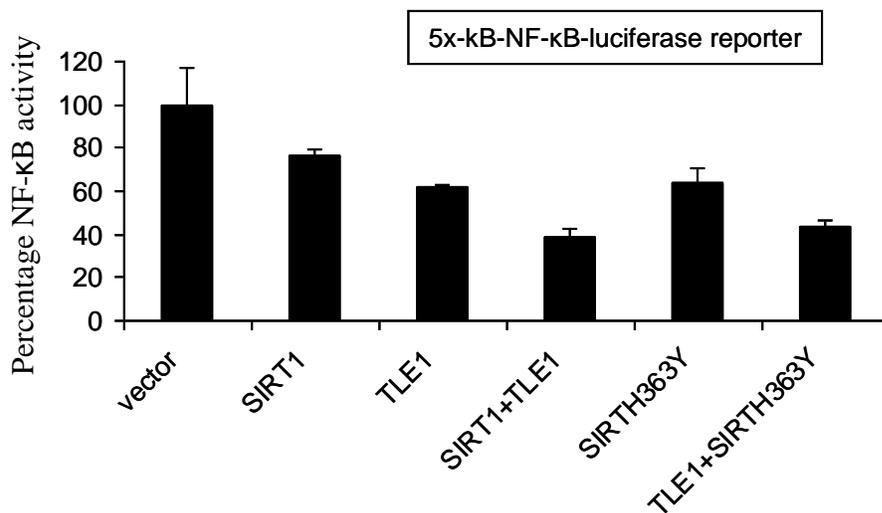


Figure 2.8 The catalytic mutant SIRT1-H363Y also represses NF- κ B activity

The data is presented as fold repression of luciferase activity. Cells were transfected with 50ng of 5x- κ B-TATA-Luc reporter plasmid and 500ng of the indicated expression plasmids. pCMV-3B, the vector control was included such that all transfections had equivalent amounts of expression plasmids. Renilla was used as the internal transfection control. 24 hr post-transfection cells were induced with TNF α for 3hr and harvested for luciferase assay.

Given that the effect of SIRT1 on NF- κ B transcriptional activity is independent of SIRT1 catalytic activity, we wished to examine which region of SIRT1 was important for conferring the NF- κ B inhibitory activity. For this we cloned the amino (1-270 a.a.) and carboxy terminal (500-747 a.a) fragments of SIRT1 in pcDNA6A expression plasmids (Figure 2.10).

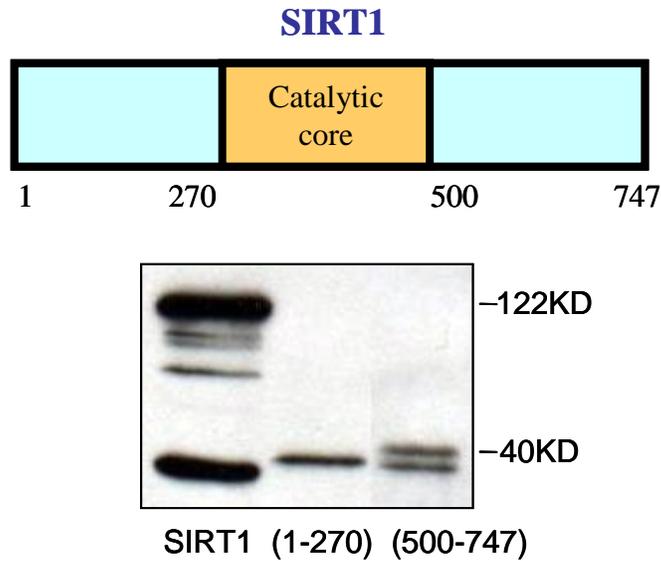


Figure 2.9 N-terminal and C-terminal Flag-tag-SIRT1

A 250 a.a stretch of N and C terminal SIRT1 was cloned in pc-DNA6A. Expression of the truncated protein was checked by western blot analysis for the 40kD fragments using Flag-tag antibody.

As shown in Figure 2.11, overexpression of the amino-terminal region of SIRT1 repressed NF- κ B activity similar to full length SIRT1 whereas the carboxy-terminal fragment of SIRT1 did not repress.

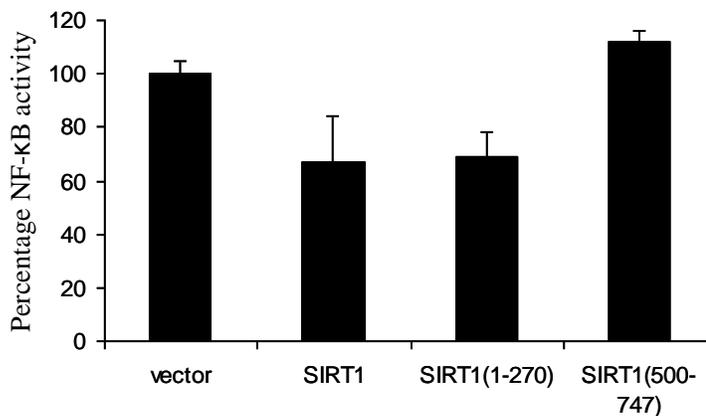


Figure 2.10 N-terminus but not C-terminus SIRT1 represses NF- κ B activity

As a further control, we examined the levels of expression of the exogenous SIRT1 and TLE1 as well as the endogenous NF- κ B subunits p65 and p50 in response to TLE1 and/or SIRT1 overexpression. As shown in Figure 2.12 A, B and C, expression of SIRT1 did not affect the levels of expression of TLE1 and TLE1 did not affect expression of SIRT1. Also, there was no effect of TLE1 and Sirt1 expression on the levels of the endogenous NF- κ B subunits.

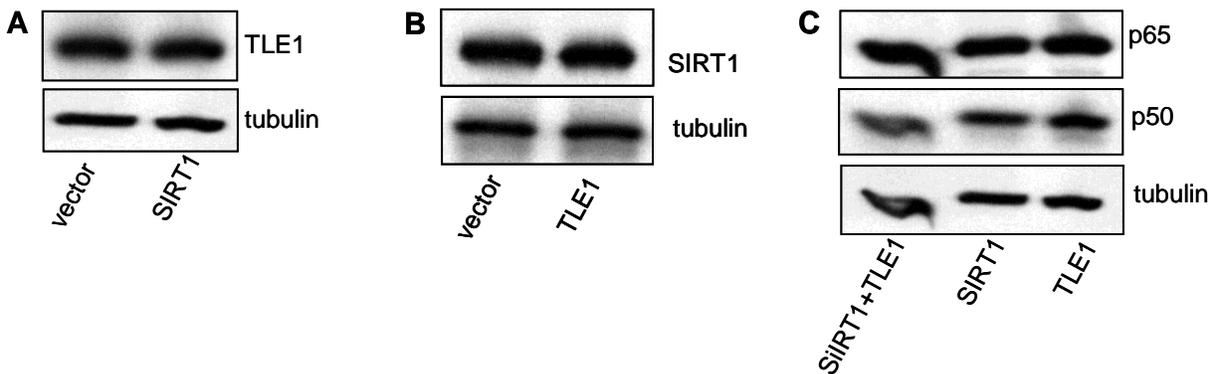
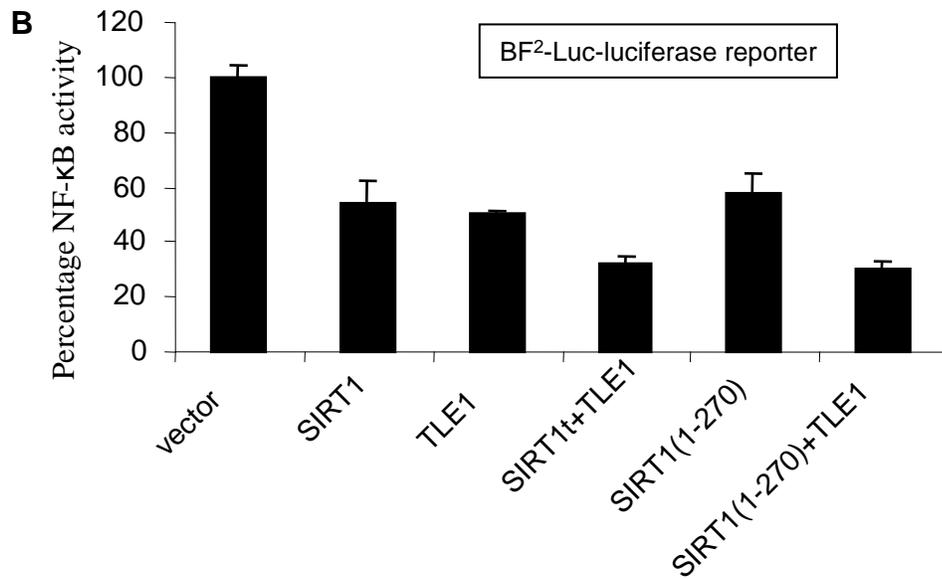
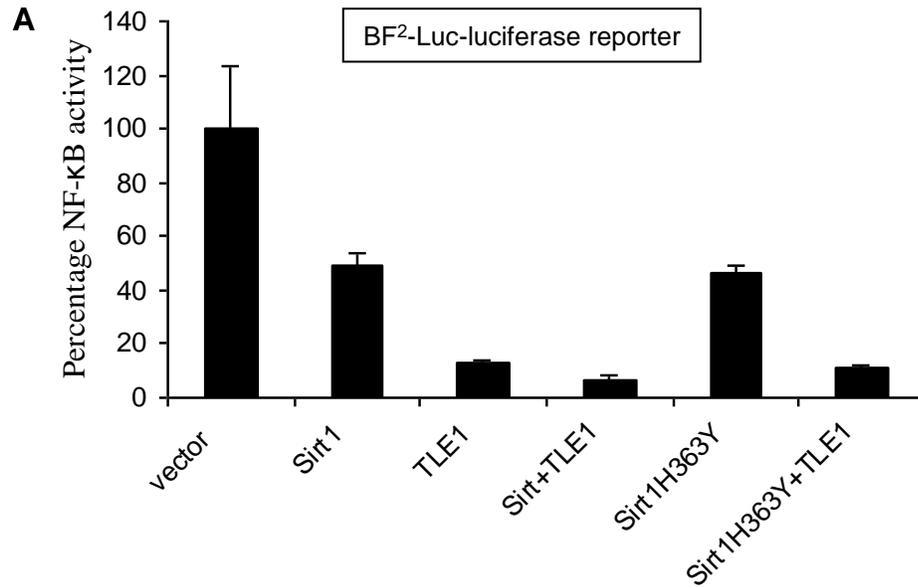


Figure 2.11 Protein levels of SIRT1, TLE1, p65 or p50 are not affected by SIRT1 or TLE1 overexpression either individually or together.

Hela cells were transfected with (A) SIRT1 or its backbone vector, (B) TLE1 or its backbone vector or with (C) TLE1 or SIRT1 or both. 24 hr post transfection the levels of TLE1, SIRT1 and p65-p50 were detected by western blot analysis. Anti-tubulin antibody was used for showing load control.

To determine if SIRT1 and TLE1 also regulate transcription from non-synthetic NF- κ B binding sites, we used a natural NF- κ B responsive luciferase reporter, BF²-Luc, containing NF- κ B sites from the IL-8 promoter. As shown in Figure 2.13, expression of either SIRT1 or TLE1 alone also was able to repress transcription from the NF- κ B sites in the IL-8 promoter (A), similar to the synthetic NF- κ B binding site reporter. Furthermore, co-expression of SIRT1 and TLE1 together repressed NF- κ B activity even more efficiently. Also, the catalytic mutant of SIRT, SIRT1H363Y (A), and the amino terminal of SIRT1 (B), which does not have the catalytic domain, were able to repress NF- κ B activity similar to the full length SIRT1. This repression was further enhanced when co-expressed with TLE1. We again show using the IL-8,

BF²-luc reporter that while the N-terminus of SIRT1 (1-270 a.a) was able to repress NF-κB activity, no repression was observed with the C-terminus (500-747 a.a) (C).



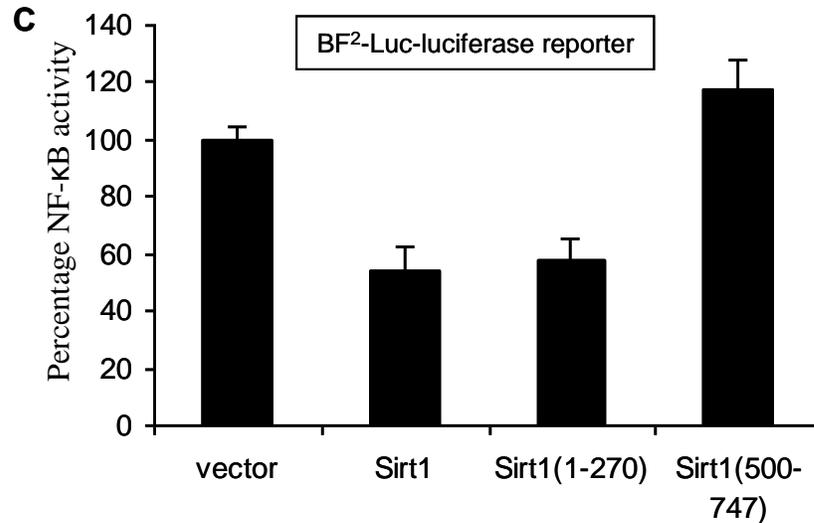
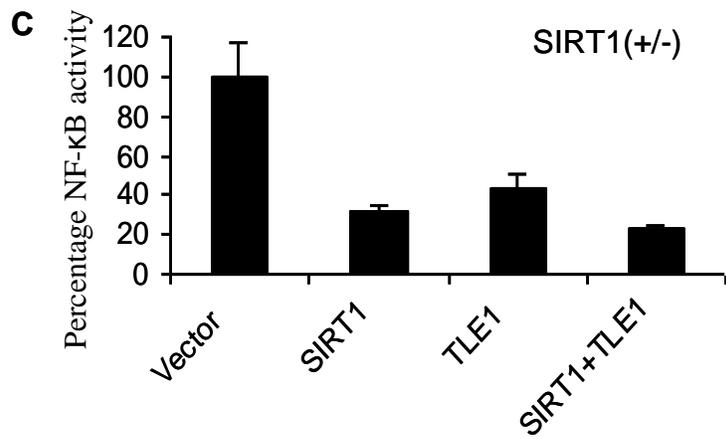
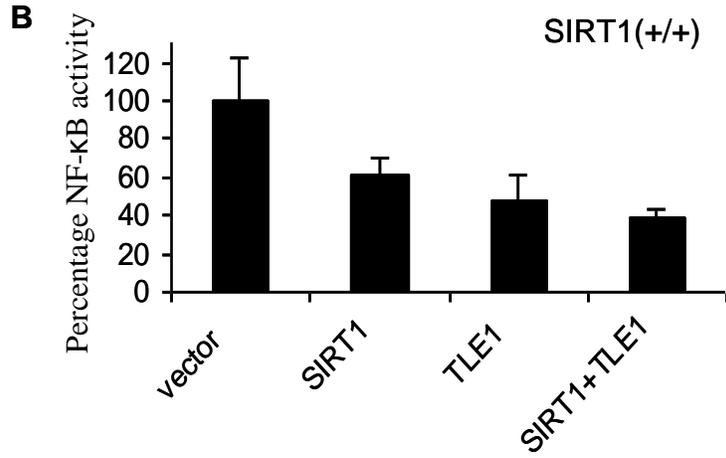
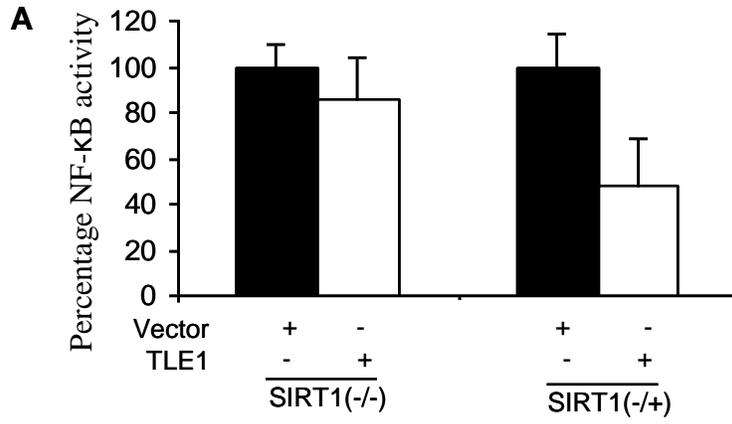


Figure 2.12 SIRT1 and TLE1 represses IL8 promoter based natural NF-κB luciferase reporter.

Cells were transfected with 50 ng of IL-8 promoter based, NF-κB responsive, BF²-Luc reporter plasmid and 500ng of the indicated expression plasmids. pCMV-3B, the backbone vector was included such that all transfections had equivalent amounts of expression plasmids. Renilla was used as the internal transfection control. 24 hr post-transfection cells were induced with TNFα for 3hr and harvested for luciferase assay.

2.4.4 SIRT1 and TLE1 are both required for inhibiting NF-κB activity

To determine if SIRT1 is required for the inhibitory effect of TLE1 on NF-κB activity, the co-transfection assays with the NF-κB reporter were performed in murine embryonic fibroblasts (MEF) that were either heterozygous null (-/+) or homozygous null (-/-) for SIRT1 [206]. As shown in Figure 2.14, TLE1-mediated repression of NF-κB transcriptional activity was significantly abrogated in SIRT1 (-/-) MEFs even though TLE1 could repress NF-κB activity by 60% in SIRT1 (-/+) MEFs (A). Furthermore, repression of NF-κB activity mediated by TLE1 was restored in SIRT1 null (-/-) MEFs by co-transfection with the SIRT1 expression plasmid. As demonstrated in Figure 2.14 B, C and D, the pattern of repression of NF-κB activity mediated by SIRT1 and TLE1 was restored in SIRT1 null MEFs by transfection with the SIRT1 expression plasmid.



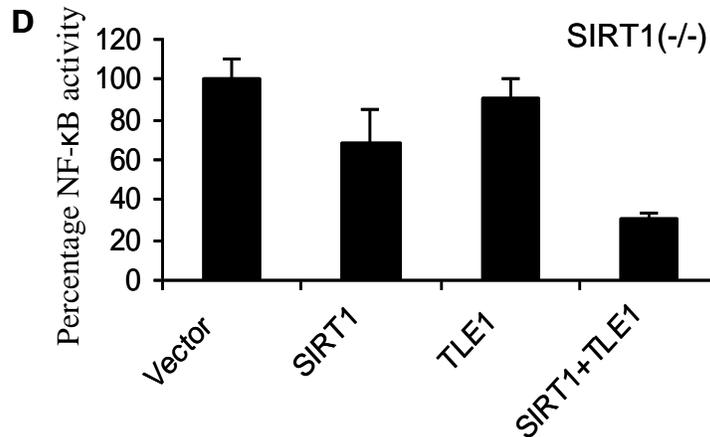


Figure 2.13 TLE1 is unable to repress NF-κB dependent gene expression in mouse embryonic fibroblasts (MEF) that are null for SIRT1

A, Murine embryonic fibroblasts that are homozygous SIRT1 null (-/-) or heterozygous SIRT1 null (-/+) were transfected with 50ng 5x-κB-TATA-Luc reporter plasmid and 500ng of the indicated expression plasmids. pCMV-3B was used as the vector control. Renilla was used as the internal control for transfection. 24 hr post-transfection cells were induced with TNFα for 3hr and harvested for luciferase assay. **B, C, and D**, In the presence of exogenously expressed SIRT1, TLE1 is able to repress NF-κB dependent gene expression in SIRT1 null MEFs. MEFs that are wild type (B), heterozygous SIRT1 null (C) or homozygous SIRT1 null (D) were transfected with 50ng 5x-κB-TATA-Luc reporter plasmid and 500ng of the indicated expression plasmids. Renilla was used as the internal control for transfection. 24 hr post-transfection cells were induced with TNFα for 3hr and harvested for luciferase assay.

The results above suggest that the presence of SIRT1 is required for the inhibition of NF-κB by TLE1. To determine if TLE1 was required for the suppression of NF-κB activity by SIRT1, HeLa cells that stably expressed shRNAi to TLE1 or to SIRT1 were established by retroviral transduction and selection as described in ‘Materials and Methods’. As shown in Figure 2.15, SIRT1 was unable to repress NF-κB in cells with reduced levels of TLE1 (A). Conversely, in cells with reduced SIRT1 levels, TLE1 was unable to mediate repression of NF-κB activity, similar to results from transfection of the SIRT1 (-/-) MEFs (B). Taken together our

data suggest that an interaction between SIRT1 and TLE1 is important for the repression of NF- κ B activity.

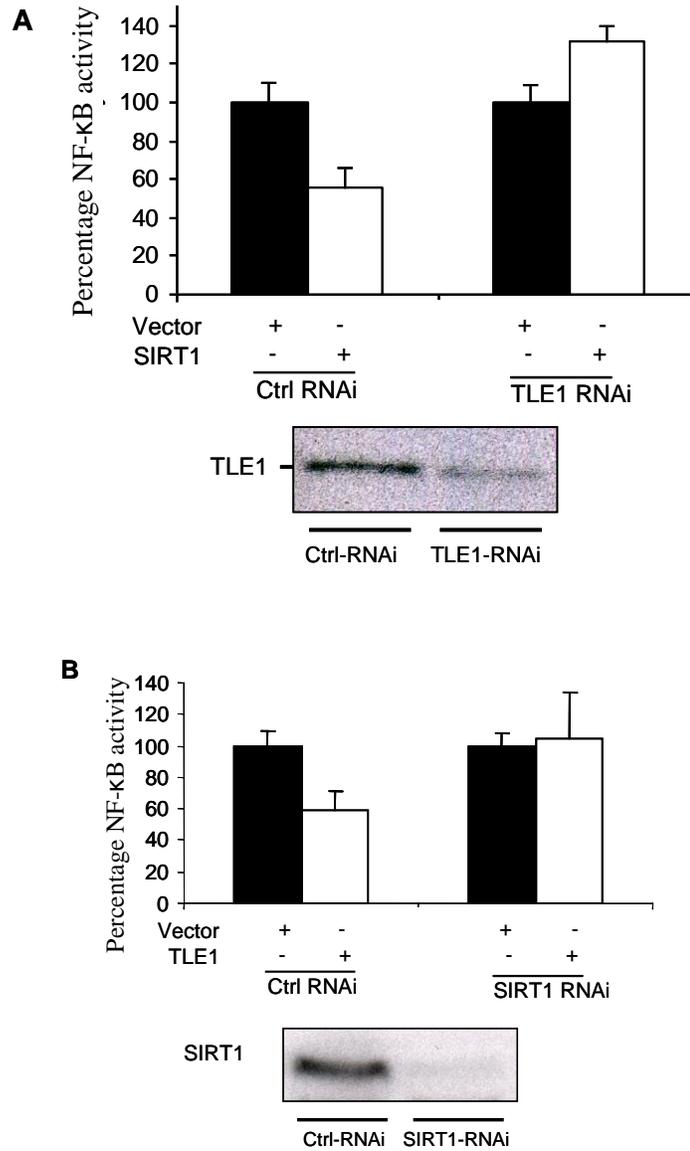


Figure 2.14 SIRT1 and TLE1 are both required for inhibiting NF- κ B activity in HeLa cells.

The data is presented as percent activity of luciferase activity in HeLa cells that were depleted of either SIRT1 (**A**) or TLE1 (**B**) using siRNA. The cells were transfected with 50ng of 5x- κ B-TATA-Luc reporter plasmid and 500ng of the indicated expression plasmids. Renilla was used as the internal transfection control. 24 hr post-transfection cells were induced with TNF α for 3hr and harvested for luciferase analysis.

2.5 DISCUSSION

SIRT1 is known to repress transcription by associating with several transcription factors such as bHLH repressor proteins, HES1, HEY2 [288] and COUP-TF interacting protein 2 (CTIP2) [270]. It also plays a direct role in repressive-chromatin formation by interacting with histone proteins H1, H3 and H4 [299]. Although the yeast Sir2 is known to deacetylate histone proteins, its human homolog, SIRT1, has been also shown to deacetylate numerous non-histone substrates including the nuclear factor κ B. SIRT1 has been shown to inhibit NF- κ B transcriptional activity by binding to and deacetylating p65/RelA [46,320]. Recently, Gao *et al.* showed that the breast cancer associated protein, BCA3, plays a role in recruiting SIRT1 to NF- κ B. Furthermore, it was shown that neddylation of BCA3 is critical for its association with SIRT1 and hence for SIRT1 mediated transcriptional regulation of NF- κ B [91]. The results presented here and other unpublished observations from our laboratory strongly suggest that SIRT1 is able to regulate NF- κ B activity through a deacetylase-independent mechanism. Overexpression of wild-type SIRT1 and catalytic mutant SIRT1, SIRT1-H363Y, both, repressed a NF- κ B dependent promoter-luciferase reporter in HeLa cells. Moreover, the N-terminal fragment of SIRT1, which does not have any catalytic activity, also repressed NF- κ B activity. Furthermore, repression mediated by SIRT1 was not abrogated by nicotinamide, an inhibitor of SIRT1 deacetylase activity (unpublished data).

In this study, we used a yeast two-hybrid screen to identify TLE1, a known repressor of NF- κ B activity, as a SIRT1 interacting factor. The association between SIRT1 and TLE1 was further confirmed by co-immunoprecipitation of both endogenous and exogenous SIRT1 and TLE1. Moreover, we showed that endogenous SIRT1 is required for TLE1-mediated repression of NF- κ B activity and, conversely, endogenous TLE1 is required for repression of NF- κ B

activity mediated by SIRT1. Taken together our results suggest that SIRT1 is able to regulate NF- κ B activity through a deacetylase independent mechanism and requires TLE1.

TLE1 is the mammalian homolog of the *Drosophila* Groucho protein, a member of a family of transcriptional co-repressors. These co-repressors lack a DNA binding domain and are recruited to DNA through their interaction with DNA binding proteins. Earlier studies on the *Drosophila* homolog of NF- κ B, Dorsal, has shown that it acts both as a transcriptional activator and repressor depending upon its interaction with other transcriptional co-activators or co-repressors [43]. For example, Groucho directly binds to Dorsal, and converts it from transcriptional activator to repressor [71]. The mammalian Groucho homologs, TLE1 and the Amino terminal Enhancer of split (AES), also directly interact with mammalian NF- κ B to repress its transcriptional activity [293]. However the exact mechanism by which TLE1 associating with p65 leads to repression of NF- κ B activity remains to be elucidated. In *Drosophila*, the recruitment of Groucho is known to be necessary, but not sufficient for Dorsal-mediated repression and requires formation of a multiprotein-DNA binding complex consisting of Dorsal, Groucho and additional proteins such as *cut* and *dead-ringer* [71]. Hence, it is likely that TLE1 may require additional proteins such as SIRT1 to mediate the repression of NF- κ B activity. Interestingly, TLE1 and SIRT1 share certain biological properties. For example, SIRT1 and TLE1 are both involved in mediating transcriptional repression by facilitating heterochromatin formation, TLE1 by virtue of it being a transcriptional co-repressor and SIRT1 by virtue of it being a histone deacetylase (HDAC-III). Both SIRT1 and TLE1 play a direct role in formation of repressive chromatin by associating with histone H3 [235,299]. Also, both of these proteins lack a DNA binding domain and are recruited to the DNA by virtue of their interaction with various DNA binding proteins and histone proteins. Furthermore, both of these

proteins have been reported to associate with the RelA/p65 subunit of NF- κ B thereby repressing NF- κ B mediated gene transcription.

Our data demonstrate that neither SIRT1 nor TLE1 are able to repress NF- κ B activity in the absence of the other. This suggests that the interaction between these proteins may play a critical role in repressing NF- κ B activity. AES binds human p65 in the vicinity of its transactivation domain [293]. Thus it is possible that TLE1 recruits SIRT1 to the transactivation domain of p65 to deacetylate lysine 310, mediating repression of NF- κ B activity. However, as described above, our data also suggests that the catalytic activity of SIRT1 may not be critical for repression of NF- κ B activity. Thus, alternatively, SIRT1 may play a role in recruiting the transcriptional co-repressor TLE1 to the p65 subunit of NF- κ B, converting it from a transcriptional activator to a repressor by associating with histone proteins or other members of the transcriptional machinery.

Our observation indicating a deacetylase independent function of SIRT1 may potentially explain some controversial data regarding SIRT1's role in cellular stress resistance through p53 decetylation. Although overexpression of mouse SIRT1 was reported to increase cell survival after exposure to hydrogen peroxide and reduce apoptosis after exposure to etoposide [198], the role of SIRT1's catalytic activity in cell survival under DNA damage or oxidative stress have been contradicted by other reports. For example, Vaziri et al showed that SIRT1 overexpression in BJ cells had no effect on cell survival after exposure to gamma irradiation [302], and more recently, work by Solomon et al, demonstrated that although SIRT1 deacetylated p53, this did not play a role in cell survival following DNA damage in certain cell lines and primary human mammary epithelial cells as inhibition of SIRT1 catalytic activity by EX-527 had no effect on cell growth, viability, or p53-controlled gene expression in cells treated with etoposide [282].

Given SIRT1's established role in increasing cellular stress resistance and the inconsistency in SIRT1's deacetylase activity in mediating cellular stress resistance, it is conceivable that SIRT1 potentially regulates the cellular resistance to stress by functioning through multiple potentially independent mechanisms, some of which may not require the deacetylase activity. Our finding demonstrates one such process that is regulated by SIRT1 potentially through a deacetylase dependent as well as independent mechanism.

Even though the exact mechanism through which the interaction between SIRT1 and TLE1 help mediate repression of NF- κ B activity is currently unclear, our results demonstrate for the first time, that the mammalian Groucho protein, TLE1, can associate with the sirtuin family protein SIRT1 to convert the transcriptional activator NF- κ B into a transcriptional repressor. This result suggests that other transcriptional factors repressed by TLE1 may also be regulated by SIRT1 and conversely, factors regulated by SIRT1 may be regulated by TLE1.

**3.0 SIRT1 ASSOCIATES WITH EUKARYOTIC INITIATION FACTOR 2-ALPHA
AND PLAYS A ROLE IN CELLULAR STRESS RESPONSE**

Hiyaa Singhee Ghosh *et al.*

3.1 ABSTRACT

SIRT1 is a NAD⁺ dependent deacetylase that plays a role in cellular stress response and cell survival. It increases lifespan in various organisms ranging from yeast, worms, flies to mice. By deacetylating p53, Ku70, E2F1, p73 and the Forkhead transcription factors, SIRT1 inhibits apoptosis and promotes cell survival. Using a yeast two hybrid screen we identified eIF2-alpha to be a potential binding partner for SIRT1. SIRT1-eIF2-alpha association was further confirmed by co-immunoprecipitation of the two proteins in human cell lines. eIF2-alpha plays a critical role in regulating the initiation step of mRNA translation in response to stress. Phosphorylation of eIF2-alpha is the first step towards shutting down global protein synthesis in response to stress stimuli, thereby facilitating preferential expression of stress related genes for effective functioning of cells under stress condition. We demonstrate that under various different stress conditions, SIRT1 depleted HeLa cells show higher phosphorylation of eIF2-alpha. Also, we observed down-regulation of cell cycle protein Cyclin D1 in SIRT1 depleted cells suggesting inhibition of global protein synthesis, and upregulation of the stress protein ATF4. We further demonstrate that SIRT1 associates with eIF2 α regardless of stress condition or its catalytic activity. Also, eIF2 α associated with SIRT1 regardless of its phosphorylation state. These observations suggest a novel aspect of SIRT1 mediated regulation of cellular stress response.

3.2 INTRODUCTION

SIRT1 is an NAD⁺ dependent deacetylase that plays a role in a wide range of cellular processes including cell proliferation, senescence and apoptosis, thereby regulating cellular stress response and lifespan. SIRT1 is believed to delay the aging process by regulating metabolism and endocrine signaling and protecting against age related diseases [4,315]. While many studies point towards a definitive role of SIRT1 in enhancing cell survival and delaying the aging processes, the mechanism by which all this is achieved is not fully clear and seems to involve a complex network of various functions of SIRT1. One of the predominant functions of SIRT1 that plays a role in longevity is regulation of cellular stress response, which protects cells from genetic and environmental damage. The wide spectrum of SIRT1's role in cell proliferative and stress resistance pathways can be attributed mainly to the regulation of FOXO proteins and p53 by SIRT1. SIRT1 regulates FOXO signaling in a complex cell and context dependent manner. It induces FOXO target stress response genes GADD45 and MnSOD while repressing the apoptotic genes, BIM and Fas [34,97,216]. SIRT1 antagonizes p53 apoptotic pathways by deacetylating the carboxy terminal of p53 which diminishes its DNA binding affinity [198,302].

In order to adapt to a myriad of physiological and environmental stresses, the cells modulate their gene expression to coordinate expression of stress-response genes, which affects cell survival, apoptosis, cell-cycle progression and differentiation. The global translation is suppressed as a response to stress in order to save the energy that is consumed during translation. However, the selective translation of genes required for cell survival under stress is activated despite of stress-induced attenuation of global protein synthesis [114,123]. The eukaryotic initiation factor 2-alpha (eIF2 α) is central to translation control in response to cellular stress. Translation is controlled by different extra and intra-cellular stimuli, such as nutrients, growth

factors, hormones and stress signals. The inhibition of protein synthesis, as a result of eIF2 α phosphorylation occurs very rapidly following exposure to stress; within 30 min of endoplasmic reticulum (ER) stress, eIF2 α phosphorylation and translation suppression is complete. This immediate action is to prevent further influx of nascent proteins into an already saturated ER lumen. Translation has three distinct phases: initiation, elongation and termination. Although all three steps have regulatory mechanisms, the rate-limiting step (initiation) is the most critical step and acts as a checkpoint in translation. Translation initiation is a complex process that begins with the interaction of the cap-binding protein complex, eukaryotic initiation factor-4F (eIF4F), with the 5'-end cap structure (m⁷GpppN, where N is any nucleotide) of the mRNA. eIF4F comprises of three components: eIF4E, which is the cap-binding protein, eIF4A, a RNA helicase, and the scaffolding protein eIF4G that bridges the mRNA and the ribosome through eIF3. 40S ribosome subunit, which associates with eIF3 and eIF1A, binds the ternary complex that consists of eIF2, methionyl-initiator tRNA (Met-tRNA^{iMet}) and GTP to form the 43S pre-initiation complex. After the recognition of the cap structure at the 5' end, the 40S subunit, as a part of the 43S pre-initiation complex, is recruited to the mRNA, which then scans the mRNA in the 5' to 3' direction until it relocates at an initiation codon, where it is joined by the 60S ribosomal subunit to form the 80S initiation complex and start translation. The initiation factors used in the formation of the initiation complex are released and recycled for a second round of initiation, in a reaction catalyzed by eIF5.

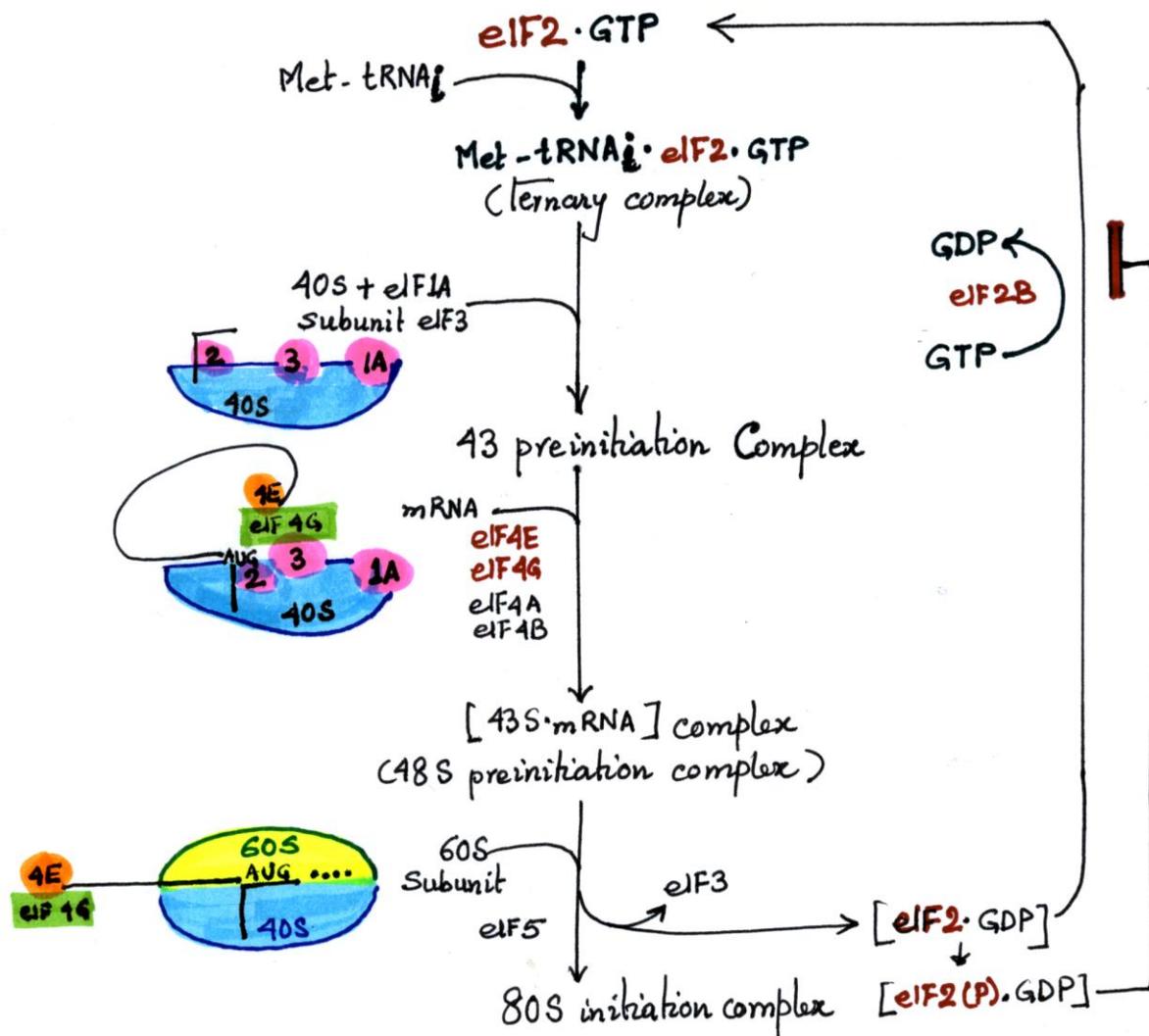


Figure 3.1 Translation initiation process of eukaryotic mRNA

The initiation processes is mediated by the eukaryotic translation initiation factors (eIFs). The formation of 43S pre-initiation complex is decided by the availability of a pool of dissociated ribosomal subunits, which is bound by eIF1A and eIF3. The 43S pre-initiation complex is formed when 40S ribosomal subunit bound to eIF1A and eIF3, binds to the ternary complex consisting of eIF2-GTP-Met-tRNA_i^{Met}. eIF3 and eIF4G associated with the cap-binding protein complex, eIF4E, then assists in recruiting the 43S pre-initiation complex to the mRNA resulting in the 48S initiation complex. The initiation factors are released after the formation of the 48S initiation complex so that they can be recycled for another round of initiation, in a reaction catalyzed by eIF5. eIF5 facilitates the

hydrolysis of the GTP carried by eIF2 resulting in dissociation from the 48S complex. Once recruited to the mRNA, 40S subunit scans from 5' to 3' on the mRNA and locates itself on the initiation codon (usually an AUG), where the 60S subunit joins to form the 80S initiation complex, thereby starting translation initiation. eIF5B is required for the joining of the 60S subunit, at which point the polypeptide elongation step starts.

eIF2 α phosphorylation regulates translation at the rate limiting initiation step. The assembly of the ternary complex (eIF2-Met-tRNA^{Met}-GTP) which is bound by the 40S subunit, is regulated by eIF2B. The binding of GTP to eIF2 is the rate-limiting step in the assembly of the ternary complex. During translation initiation, the GTP bound to eIF2 is hydrolysed to GDP. Before eIF2 can be recycled for another round of initiation, the exchange of the GDP bound to eIF2 with a GTP, a reaction catalyzed by eIF2B, is critical for translation to continue. Four different eIF2 α kinases, phosphorylate eIF2 α in response to various stress stimuli. General control non-derepressable-2 (GCN2) in response to amino acid starvation or UV irradiation, protein kinase RNA (PKR) in response to viral infection, heme-regulated inhibitor kinase (HRI) in response to lower heme levels, osmotic or heat shock and PKR-like ER kinase (PERK) in response to ER (endoplasmic reticulum) stress or hypoxia causes phosphorylation of the alpha subunit of eIF2 at residue serine 51.

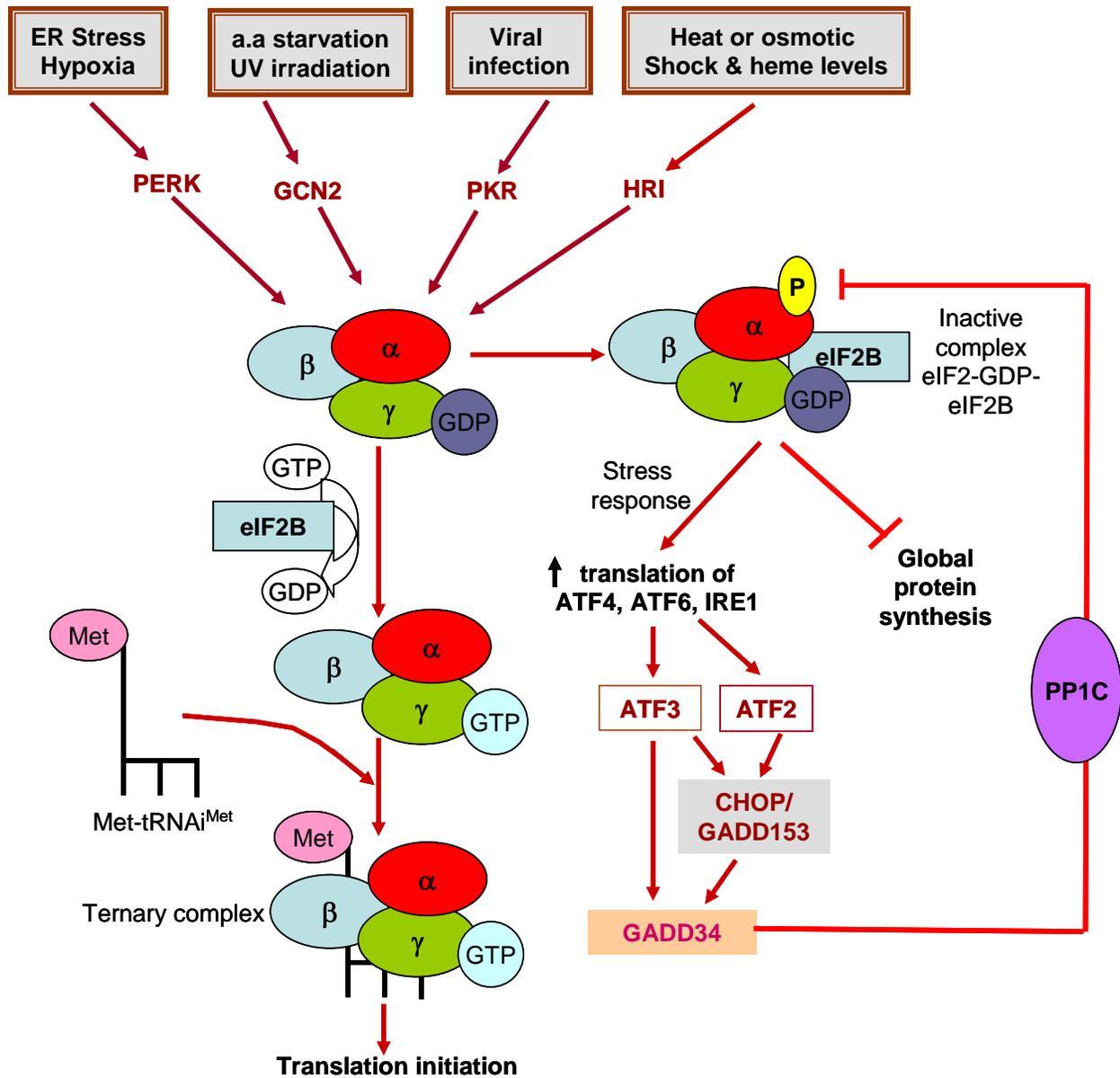


Figure 3.2 Stress stimuli mediated phosphorylation of eIF2 α inhibits translation.

The eukaryotic initiation factor eIF2 consists of three subunits; eIF2 α , eIF2 β and eIF2 γ , which form a part of the translation initiation ternary complex; eIF2-GTP-methionyl-initiator-tRNA (Met-tRNA^{iMet}). Four different kinases; GCN2, PKR, HRI and PERK respond to different stress stimuli and phosphorylate the alpha subunit of eIF2. This results in inhibition of the GDP-GTP exchange by formation of a stable and inactive eIF2-GTP-eIF2B complex. Recharging of eIF2 after the first round of initiation is accomplished by GDP-GTP exchange on eIF2, which is catalyzed by eIF2B. Thus, eIF2 α phosphorylation results in inhibition of global protein synthesis. However,

translation of selective mRNA continues which allows cells to adapt to stress conditions. Downstream effectors of the stress response act in a negative feedback loop to dephosphorylate eIF2 α for translation recovery.

Phosphorylation of eIF2 α in response to cellular stress inhibits the exchange of eIF2-GDP to eIF2-GTP required to bind the Met-tRNA_i^{Met}, thereby preventing formation of the ternary complex and inhibiting translation. Since eIF2 α is present in excess over eIF2B, small changes in the phosphorylation of eIF2 α affects the formation of ternary complex and translation significantly. Thus eIF2 α phosphorylation reduces translation of most mRNAs by preventing the recognition of 5' cap structure and translation initiation. However, eIF2 α phosphorylation selectively enhances translation of specific mRNAs that help in adaptation to stress and recovery of translation for cellular survival. This includes the stress responsive genes such as activating transcription factor 4 (ATF4), CHOP and GADD34.

The unconventional mechanisms by which specific genes escape translation attenuation in response to eIF2 α phosphorylation involve processes which do not require the 5' cap structure of mRNA for the initiation process. These mechanisms depend on regulatory regions in the 5' and 3' untranslated regions (UTR) of the specific mRNAs. Such regulatory regions are estimated to be present in as many as half of the vertebrate mRNA. Initiation of these specific mRNA involve either the 'internal ribosome entry site' (IRES) [75,76], or small upstream open reading frame (uORF) or both [210,240]. ATF4, the downstream effector of stress response, circumvents the translational block by eIF2 α phosphorylation because of the presence of two upstream open reading-frames (uORFs) in its 5' untranslated regions (UTR), one (uORF2) of which overlaps with the ATF4 ORF and inhibits ATF4 translation [113]. These uORFs, which ordinarily prevent translation of the true ATF4 ORF, are bypassed only when eIF2 α is

phosphorylated; making sure that ATF4 is made only under stress conditions. When the ternary complex for translation initiation is abundant (when eIF2 α is low), the ribosomes re-initiate at the inhibitory uORF2, resulting in translation attenuation of ATF4. Under stress, eIF2 α is phosphorylated resulting in lower eIF2-GTP levels, which in turn results in lower ternary complex availability and slowing down of re-initiation process. Ribosomes that bind at uORF1 are able to scan through uORF2 without reinitiating due to delayed initiation process, and instead initiate at the ATF4-coding region resulting in ATF4 translation only under stress condition [250,301]. Induced *ATF4* expression occurs predominantly via translation control as shown by preferential *ATF4* mRNA association with polysomes that occurs during stress-induced eIF2 phosphorylation [113]. Upregulated ATF4 levels induces a cascade of transcriptional regulators including CHOP/GADD153 and ATF3, resulting in stress gene expression important for cellular metabolism, the redox status of the cell, and apoptosis [112,115,144,153]. Downstream to ATF3/CHOP is GADD34, whose expression correlates temporally with eIF2 α dephosphorylation late in the stress response. GADD34 is a stress-inducible regulatory subunit of a holophosphatase complex that dephosphorylates eIF2 α , and plays a role in translational recovery through feed back inhibition of eIF2 α phosphorylation [59,225]. eIF2 α phosphorylation increases translation of ATF4 which acts to upregulate the transcription of CHOP, which in turn, increases GADD34 levels, late in the stress response pathway. GADD34 associates with type 1 protein phosphatase (PP1) catalytic subunit and activates it, resulting in dephosphorylation of eIF2 α which is thought to dampen the gene expression pathway when there is a sufficient level of stress gene expression. Under severe stress conditions, CHOP mediates the pro-apoptotic function of eIF2 α phosphorylation. While ectopic overexpression of CHOP results in increasing

apoptosis in HeLa cells, 3T3 fibroblasts and keratinocytes [205], CHOP deletion in MEFs have been shown to impede apoptosis [143].

Phosphorylated eIF2 α thus integrates diverse and seemingly unrelated forms of stress, to initiate signaling in a common downstream stress-response pathway, referred to as an integrated stress response.

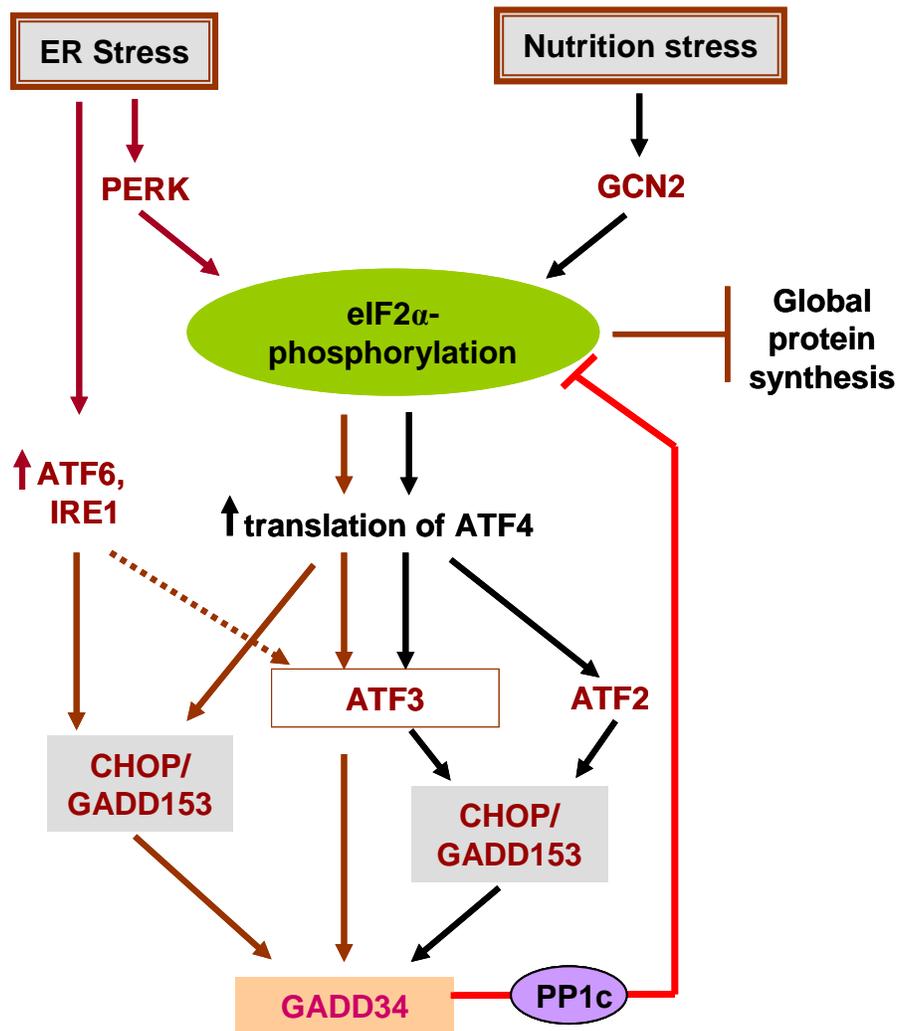


Figure 3.3 Integrated stress response by eIF2 α phosphorylation

In response to ER stress, eIF2 α phosphorylation by PERK induces ATF4 expression, which is required for increased expression of ATF3 and other stress genes. ATF3 is required for increased levels of GADD34, which directs the

type 1 protein phosphatase (PP1) to dephosphorylate eIF2 α . ER stress is also recognized by membrane-associated ATF6 and IRE1, which function to activate the unfolded protein response (UPR), including *GRP78* expression. ATF6 and IRE1 are proposed to work in conjunction with the eIF2 kinase stress pathway to enhance the expression of many stress genes, including *CHOP/GADD153* and *ATF3*. During nutritional stress, the eIF2 kinase GCN2 induces the sequential expression of bZIP transcription factors ATF4, ATF3, and CHOP. ATF3 contributes to increased *GADD34* expression, which contributes to feedback dephosphorylation of eIF2 α . Transcriptional activation by ATF3 may be indirect through protein-protein interactions. ATF4 is suggested to directly regulate stress gene expression during nutrient limitation. Analysis of MEF cells carrying a deletion of ATF2 indicate that this bZIP transcription factor is also required for *CHOP* expression in response to amino acid deprivation

eIF2 α phosphorylation also leads to activation of P13K/mTOR pathway [154], and I κ B degradation dependent and independent activation of NF- κ B activity [145]. Recently, it has been shown that the eIF2 α kinase PKR acts upstream of PI3K to turn on the Akt/PKB-FRAP/mTOR pathway leading to S6 and 4E-BP1 phosphorylation. Interestingly, PI3K signaling activation is indirect and requires the inhibition of protein synthesis by eIF2 α phosphorylation [154].

NF- κ B (nuclear factor- κ B) is an important component of the cellular stress response. UV-C and UV-B irradiation activates NF- κ B in murine embryo fibroblasts through a mechanism requiring eIF2 α phosphorylation. GCN2 and PERK phosphorylate eIF2 α in response to UV radiation. Lowered protein synthesis accompanied by eIF2 α phosphorylation, combined with eIF2 α kinase-independent turnover of I κ B (inhibitor of κ Ba), reduces the levels of I κ Ba in response to UV irradiation, resulting in release of NF- κ B from the inhibitory I κ Ba and facilitating NF- κ B entry into the nucleus and targeted transcriptional control. Thus, eIF2 α phosphorylation provides resistance to apoptosis in response to this environmental insult [142]. The mechanisms by which eIF2 α phosphorylation modulates NF- κ B activity can vary depending

on the cellular stress condition and the participation of additional stress-response pathways. For example, activation of NF- κ B in response to ER stress involves the release of I κ Ba from NF- κ B without measurable reductions in I κ Ba levels [145]. Inhibition of ubiquitin-mediated degradation significantly decreases activation of NF- κ B in response to UV or TNF α exposure, but does not diminish NF- κ B induction in response to ER stress, indicating the differences between the mechanisms activating NF- κ B during ER stress and in response to UV or TNF α treatments. Ubiquitin-mediated turnover of I κ Ba, which would contribute to lowered levels of this inhibitory protein, is not central to modulation of NF- κ B activity in response to ER stress.

SIRT1 plays a role in cell survival and apoptosis in response to stress and energy state. In an effort to find novel binding partners for SIRT1 using a yeast two-hybrid screen, we discovered that SIRT1 potentially interacts with eIF2 α . We confirmed this interaction by co-immunoprecipitation of these proteins in human cell lines. In this chapter we demonstrate that SIRT1 potentially plays a role in cellular stress response through a novel pathway involving eIF2 α . Under various stress conditions, SIRT1 depleted HeLa cells show higher phosphorylation of eIF2 α which is accompanied by reduced levels of the cell-cycle protein Cyclin-D1 and increase levels of the stress response protein ATF4. In addition, our results show that SIRT1 associates with eIF2 α regardless of stress condition. Also, neither SIRT1's catalytic activity nor the phosphorylation state of eIF2 α was important for this association, did not affect SIRT1-eIF2 α association either. Consistent with the higher phosphorylation of eIF2 α in SIRT1 depleted cells, we observed more sensitivity to oxidative stress and higher NF- κ B activity in response to ER stress. These results suggest a novel regulatory role for SIRT1 in eIF2 α mediated cellular stress response.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture and drug treatment

HeLa cells were purchased from American tissue culture consortium (ATCC) and cultured in DMEM media (Dulbecco's modified Eagle's media, Cellgro #) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100U penicillin, 100U streptomycin and 0.25 μ g/ml amphotericin B (antibiotic-antimycotic; GIBCO # 15240-062), HEPES buffer and 2mM L-glutamine. Cells were cultured at 37⁰C, 19% O₂ and 5% CO₂. The SIRT1-wild-type and SIRT1 null mouse embryonic fibroblasts were a gift from Dr. Michael McBurney, University of Ottawa, Canada. These cells were also maintained in the DMEM growth media. SIRT1 MEFs were cultured at 37⁰C, 15% O₂ and 5% CO₂. For various stress treatments, cells were seeded at about 80% confluency in 60mm culture dishes and cultured for overnight under conditions as mentioned above. Next day, cells were treated for indicated time periods in stress media such as: leucine negative media (RPMI 1640, Cellgro, # 10-040-CV), glucose negative media (DMEM), serum negative media (Normal growth media without FBS), 2 μ g/ml tunicamycin, TM (SIGMA # T7765) in normal growth media, 200nM thapsigargin, TG (SIGMA # T9033) in normal growth media, and 500 μ M H₂O₂ in normal growth media. The solvent for chemicals like TM and TG were used in similar concentration for mock-treatment where ever possible. Cells were harvested by treating cells with trypsin-EDTA (Cellgro, # 25-053-CI) for 2mins in 37⁰C incubator. Cells were washed by re-suspending cells in growth media and centrifuged at 1000g in a table-top centrifuge.

3.3.2 Transfections and Luciferase assays

Cells were seeded at ~80% confluency in DMEM growth media without antibiotic-antimycotic, and cultured overnight. Cells were transfected the next day using Lipofectamine2000 transfection reagent (Invitrogen # P/N 52887) according to manufacturer's protocol. The transfection media on cells was replaced with normal growth media 6hrs post-transfection. The next day, cells were treated as required for specific experiments and harvested for analysis. For luciferase assay, cells were seeded in 24 well plate and transfected with 100ng 5X- κ B-luciferase reporter plasmid (Stratagene) along with 100ng renilla luciferase reporter, pRL-TK plasmid (Promega) per well. 24 hrs post-transfection, cells were treated either with 10ng/ml TNF- α (R&D System) or indicated dose of thapsigargin (SIGMA # T9033) for 6hrs. Cells were harvested, followed by protein extraction using NP40 lysis buffer. Luciferase assays were performed using the Luciferase Assay System (Promega). Renilla luciferase activity expressed from the pRL-TK plasmid (Promega) was used as an internal control for transfection efficiency. 20 μ l aliquot of each sample was mixed with 100 μ l of luciferase assay reagent (Promega), and luciferase activity was measured in a Beckman Coulter LS6500 scintillation system in the single-photon mode. The data was calculated as percentage luciferase activity (mean \pm S.E) of three independent transfections relative to the control (mock-treatment).

3.3.3 Detection of SIRT1-eIF2 α association

Yeast two-hybrid assay was performed as described in Chapter 2. The interaction between SIRT1 and eIF2 α was confirmed by co-immunoprecipitation assay as described below.

3.3.4 Whole cell lysate preparation and protein estimation

Whole cell lysates were made by lysing cells in NP-40 lysis buffer (20mM TrisHCL, pH8.0, 137mM NaCl, 10% glycerol, 1% nonidet P-40, 2mM EDTA). 1X protease inhibitor cocktail (SIGMA # P2714) was added to lysis buffer just before lysis. Cells were lysed for 20 min on ice and lysate was separated from cell debris by centrifugation for 20 min at 13000g in a microcentrifuge at 4°C. Protein concentration of the cell lysates were estimated by Bradford assay. 2µl of lysate was added to 1ml BioRad protein assay reagent (#500-0006, BioRad Labs, Hercules, CA). 0, 1, 2, 3, 4, 6 and 8 µg BSA (SIGMA # A2153) from 1mg/ml stock was used to plot the standard curve. Readings were taken at 595nm wavelength.

3.3.5 Co-immunoprecipitation assay

For exogenous expression of epitope-tagged proteins, 10 µg of the pcDNA-flag-SIRT1 and pCMV-myc-eIF2α expression plasmids were transfected in HeLa cells using Lipofectamine™2000 reagent (Invitrogen # P/N 52887) as per manufacturer's protocol. 24hr post-transfection, whole cell lysates were made as described above. For immunoprecipitation, 500 µg of cell lysate was incubated with 2µg of specific protein antibody or epitope-tag antibody, overnight at 4°C on a rotator. For negative control, rabbit normal IgG antibody was used. Next day, samples were incubated with 35µl ProteinA-sepharose (SIGMA # P9424) and 25µl ProteinG-agarose (SIGMA # P4691) 50% slurry at 4°C on a rotator for 2 hrs. The resin was pelleted by spinning the samples at 2000g for 5 min in microcentrifuge. The precipitated resin was then washed three times in cold lysis buffer. Centrifugation during washes was carried at 2000g for 5 min. After the final

wash, the supernatant was carefully discarded by suction using a fine tip pipette. The beads were then resuspended in 25 μ l of 2X-Lamml sample-loading buffer (BioRad # 161-0737) and boiled for 5 min to elute the proteins. Samples were spun at highest speed for 1 min in the microfuge and supernatants were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) on 12% or 8% gels followed by western blot analysis. Antibodies used for immunoprecipitation were as followed: anti-SIRT1 (Upstate # 05-707), anti-eIF2 α (sc-11386), anti-flag-tag (Chemicon # MAB3118) and anti-myc-tag 9E10 (Upstate # 05-419), Rabbit normal IgG (SantaCruz biotech # sc2027).

3.3.6 Western blotting

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the experiment, immunoprecipitated samples or 50 μ g lysate protein were loaded on 12% or 8% polyacrylamide gels. Separating gels were made using Protogel 30% w/v acryamide: 0.8% w/v bis-acrylamide (National Diagnostic # EC-890), 1.5M Tris-HCL pH8.8, 10% ammonium persulfate (APS), 10% SDS and 0.1% TEMED (BioRad # 161-0801). 4% Stacking gel was made using same above reagents, except 1.5M Tris-HCL pH 6.8 instead of pH 8.8. Proteins of molecular weight ranging between 30-75 kD were resolved on 12% separating gels, whereas protein with higher molecular weight were resolved on 10 or 8 % separating gels. Gels were run at 10mA for one minigel in 1X-SDS-running buffer. Precision plus dual color protein marker (BioRad # 161-0374) was used as molecular weight standard. Samples resolved on SDS-PAGE were transferred on PVDF membrane (Immobilon-P, Millipore, Bedford, MA) in a semidry transfer machine (Bio-Rad, Richmond, CA) at 15V for 1-1.5 hr depending on molecular weight of the protein to be probed. Membranes were then incubated in blocking buffer (1X PBS, 5%

w/v non-fat dry milk, 1% Tween-20) for 1hr at RT on a rocker. After blocking, membranes were washed three times with wash buffer, PBST (1X PBS, 1% Tween-20) for 5min each. The washed membranes were then incubated with primary antibody overnight at 4⁰C on a rocker. Primary antibody dilutions used were as follows: 1:1000 anti-eIF α and 1:2000 for anti-SIRT1, anti-flag-tag and anti-myc-tag antibody. Following primary antibody staining, membranes were given three washes with wash buffer (PBST), 10 mins each and incubated with corresponding HRP-conjugated-secondary antibody (1:5000) for 1 hr at RT on a rocker. Membranes were then washed three times in PBST and proteins were visualized by enhanced chemiluminescence (# NEL103, PerkinElmer Life Science, Inc). For re-probing the same blot, membranes were incubated in RestoreTM western stripping buffer (Pierce # 21059) for 10-15min, followed by three washes, 10 mins each in PBST. Blots were then blocked as mentioned above and probed with appropriate primary and corresponding secondary antibody, and visualized as described above. Antibodies used for western blotting are as follows: anti-SIRT1 (Upstate # 05-707, 1:2000), anti-eIF2 α (SantaCruz Biotech # sc-11386, 1:1000), anti-flag-tag (chemicon # MAB3118, 1:2000) and anti-myc-tag 9E10 (Upstate # 05-419, 1: 2000), anti-ATF4/CREB2 (# sc-200, 1:500, 2hr at RT), anti-GADD153/CHOP (sc-575, 1:500, 2hr at RT), anti-CyclinD1 (Cell signaling #), anti-PARP1 antibody (# sc-25780, 1:5000) and anti-HSP90 (sc-13119, 1:5000).

3.3.7 Cell survival assay

Cells (10,000) were seeded per well in 100 μ l growth media in a 96 well plate and cultured overnight as mentioned above. Next day, the growth media was replaced with treated media as indicated and cells were put back in incubator for overnight. 24hr post-treatment, 10 μ l of MTT

reagent, 5mg/ml (MTT assay kit, ATCC # 30-1010K) was added to each well and incubated at 37°C for 4hr. When blue coloration was visible in mock-treated live cells, the reaction was stopped by incubating in detergent reagent for 2hr at RT in dark. The absorbance was measured in a plate reader at 570nm wavelength. The data was calculated as percentage survival (mean ± S.E) of three independent samples for each set, relative to the control (mock-treatment).

3.3.8 Immunofluorescence Microscopy

HeLa cells were seeded on sterilized (in 99% ethanol) coverslips at 60 % confluency in a 6 well plate and cultured in normal growth media for overnight. Cells were fixed in 2% paraformaldehyde for 15 min at room temperature. Immunostaining was performed by incubating fixed cells in 1:1000 diluted anti-SIRT1 (mouse, clone 2G1/F7, Upstate) antibody for 1hr, followed 45 min incubation with secondary antibody conjugated with alexa-fluor488 (anti-mouse) at RT. Samples were examined by using an Eclipse E600 microscope (Nikon). Photomicrographs were taken at 40X magnification.

3.3.9 Protein synthesis assay by ³⁵S-methionine incorporation

SIRT knock-out and wild-type mouse embryonic fibroblasts were seeded in 6 well plates at a density of 4.5×10^5 cells/well. Cells were stress treated for 1hr with 2 μ M thapsigargin. Cells were incubated in normal growth media for the rest of the time after 1 hr stress treatment. 30min prior to the specific time points, cells were depleted for methionine and cysteine by replacing the media with methionine/cysteine negative growth media (Gibco DMEM –methionine and –cysteine # 21012). At specific time points, cells were pulsed with 200 μ Ci/ml ³⁵S-protein labeling mix

(Easy-tag Express ³⁵S-protein labeling mix, Perkin-Elmer#NEG772014MC). Cells were incubated at 37°C incubator for 30 min after adding the radioactivity. Cells were then harvested and lysed for western blot analysis. Radioactivity was detected by exposing the gel, wrapped in saron wrap, to Kodak BioMax MR film.

3.4 RESULTS

3.4.1 SIRT1 interacts with eIF2 α *in vivo*

To identify potential binding partners for SIRT1, we used a yeast two-hybrid system to screen human cDNA library, using SIRT1 fused to the Gal4-DNA binding domain (DBD) as the bait. We screened approximately 10.5×10^6 transformants of a human spleen cDNA library fused to the GAL4 activation domain (AD) in the AH109 yeast strain as described in [94]. The identification of eIF2 α as a potential binding partner for SIRT1 in the our yeast two-hybrid screen was interesting because both SIRT1 and eIF2 α play a role in cellular stress response.

To further confirm this interaction in human cells, we performed co-immunoprecipitation assays for these two proteins in HeLa cells. Endogenous SIRT1 and eIF2 α as well as transiently expressed, epitope-tagged versions of the proteins were examined for co-immunoprecipitation. Immunoprecipitation of whole cell extracts from HeLa cells with anti-eIF2 α or anti-SIRT1 antibodies followed by western blot analysis with anti-SIRT1 or anti- eIF2 α antibody respectively, demonstrated that the endogenous SIRT1 and eIF2 α proteins interact under physiological conditions (Fig. 3.4 A and B). Consistent with this observation, flag-tagged SIRT1 and myc-tagged eIF2 α were also co-immunoprecipitated from transfected HeLa cells (Fig. 3.4 C and D).

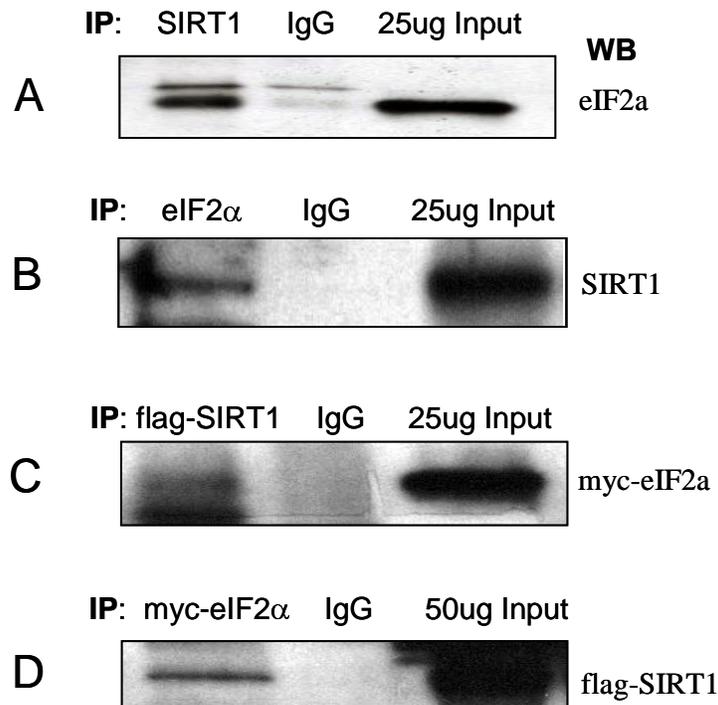


Figure 3.4 SIRT1 and eIF2 α interact with each other in HeLa cells

A. Whole cell extracts from HeLa cells were immunoprecipitated for SIRT1 or eIF2 α using their respective antibodies. Samples were then probed for eIF2 α and SIRT1 by western blotting. B. HeLa cells were transfected with flag-tagged SIRT1 and myc-tagged eIF2 α . 24hr post transfection, whole cell extracts were immunoprecipitated for SIRT1 or eIF2 α using flag or myc antibodies respectively, followed by western blot analysis using myc and flag antibodies respectively. IgG antibody was used as a negative control for immunoprecipitation and 25-50 μ g of whole protein lysate was used as input.

3.4.2 SIRT1 localizes in the cytoplasm of human and mouse cells

SIRT1 is predominantly known as a nuclear protein. However, several recent reports have not only demonstrated a cytoplasmic presence of SIRT1 but also have identified certain cytoplasmic functions of SIRT1 [55,217,289]. Interestingly, in our laboratory, we observed that SIRT1 was

both a nuclear and cytoplasmic protein. Using immuno-fluorescence for SIRT1, we detected cytoplasmic localization of SIRT1 in HeLa cells (Figure 3.5).

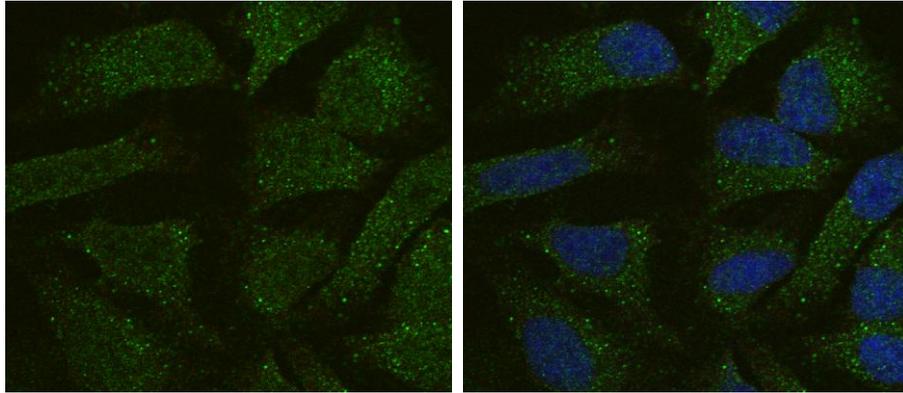


Figure 3.5 SIRT1 localizes in the nucleus and cytoplasm of HeLa cells

HeLa cells were probed with anti-SIRT1 antibody (green) and localization of SIRT1 was detected by immunofluorescence microscopy. The nucleus was stained with DAPI (blue). Pictures were taken at 40X magnification.

To confirm the cytoplasmic presence in different mammalian cell types, we analyzed SIRT1 by sub-cellular fractionation of three different human cell lines and mouse embryonic fibroblasts. As shown in Figure 3.6, SIRT1 localizes in the nucleus as well as the cytoplasm of HeLa, 293T, Jurkat cell lines and in mouse embryonic fibroblasts (MEFs). Notably, we found more cytoplasmic than nuclear SIRT1 in each of these cell lines. The cytoplasmic localization of SIRT1 was relevant to its association with eIF2 α , because eIF2 α is a translation regulatory protein and translation is a cytoplasmic process. Hence, SIRT1's cytoplasmic localization and its association with eIF2 α , together indicated towards a cytoplasmic function of SIRT1, which could be potentially, mediated through eIF2 α .

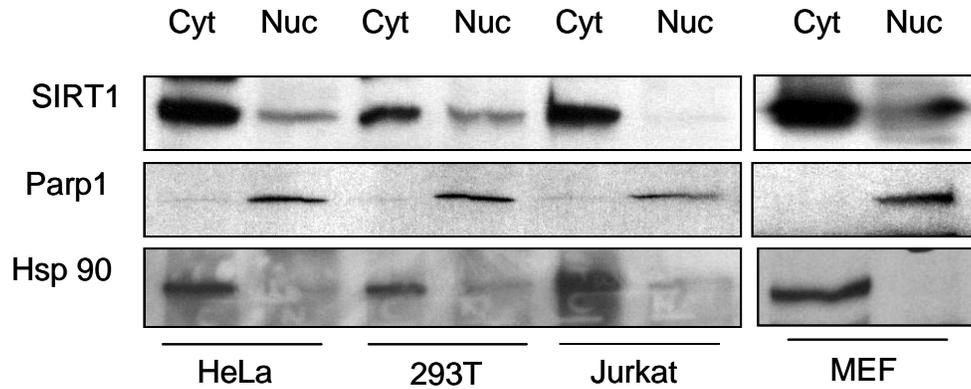


Figure 3.6 SIRT1 localizes in the cytoplasm and nucleus of human and mouse cell lines

Sub-cellular fractions of the indicated cell lines were examined by western blot analysis. Parp1, a nuclear protein, was used as a positive control for nuclear fractionation whereas Hsp90, a cytoplasmic protein, was used as a positive control for cytoplasmic fractionation. ‘Cyt’: cytoplasmic, ‘Nuc’: Nuclear.

3.4.3 eIF2 α phosphorylation is enhanced in SIRT1 depleted cells

Given that SIRT1 plays a role in stress resistance and longevity and our finding that SIRT1 interacts with eIF2 α in human cells, we wished to investigate the possible regulatory overlap between these two proteins in eIF2 α mediated stress response. For this, we used SIRT1 depleted HeLa cells made by stable retroviral expression of shRNAi against SIRT1 [94]. These cells were treated for 1 hr with different stress conditions, such as amino-acid starvation, glucose starvation, serum deprivation, ER stress (tunicamycin) and oxidative stress (H₂O₂). As shown in Figure 3.7 A, under all different stress conditions, the SIRT1 depleted cells showed higher levels of phosphorylated eIF2 α than the matched control cells, indicating SIRT1’s role in stress dependent regulation of eIF2 α phosphorylation.

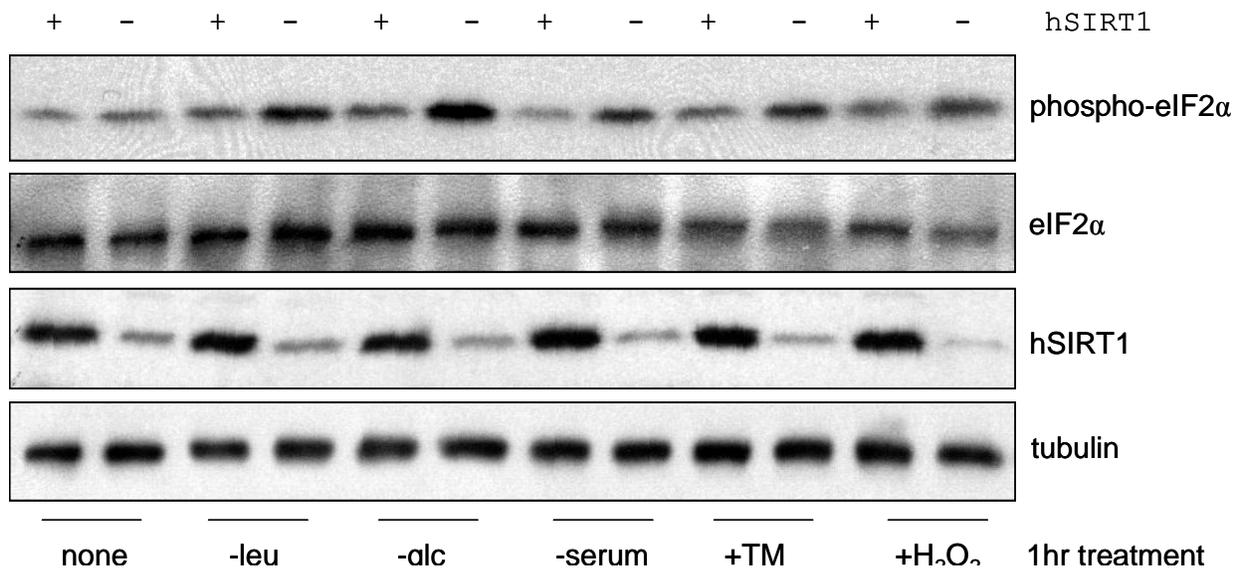


Figure 3.7 HeLa cells depleted for SIRT1 show higher eIF2 α phosphorylation in response to stress

SIRT1 depleted HeLa cells (generated by stable retroviral expression of shRNAi for SIRT1) and the matched control cells were treated for 1hr as indicated. Whole cells lysates were analysed by western blotting using phosphorylated and total protein antibodies for eIF2 α . The blot was also probed for SIRT1 to show efficient knock-down of SIRT1 protein. Tubulin was used as a loading control for the blots. -leu: leucine starvation, -glc: glucose starvation, -serum: serum starvation, +TM: 2 μ g/ml tunicamycin, H₂O₂: 500nM hydrogen peroxide, hSIRT1: human SIRT1.

3.4.4 SIRT1 depleted cells show upregulated eIF2 α phosphorylation for a prolonged time

Next, we investigated the kinetics of eIF2 α phosphorylation between the two types of cells; SIRT1 depleted and matched control HeLa cells. Control and SIRT1 depleted HeLa cells were treated with two different stress conditions: amino acid starvation (-leucine) and ER stress (thapsigargin: causes depletion of ER-Ca²⁺) for up to 8hrs and the phosphorylation of eIF2 α was analyzed by western blotting. Interestingly, compared to the control cells, the phosphorylation of eIF2 α was higher in SIRT1 depleted cells at all times points, suggesting that the role of SIRT1 in

eIF2 α phosphorylation is not time dependent (Figure 3.8). Also, while the control cells showed an overall reduction in eIF2 α phosphorylation in the later stage of stress (8h), the SIRT1 depleted cells showed sustained phosphorylation of eIF2 α through all time points. This suggests that SIRT1 may be involved in mediating recovery processes during the later phase of stress response which is important for translation recovery and cell survival.

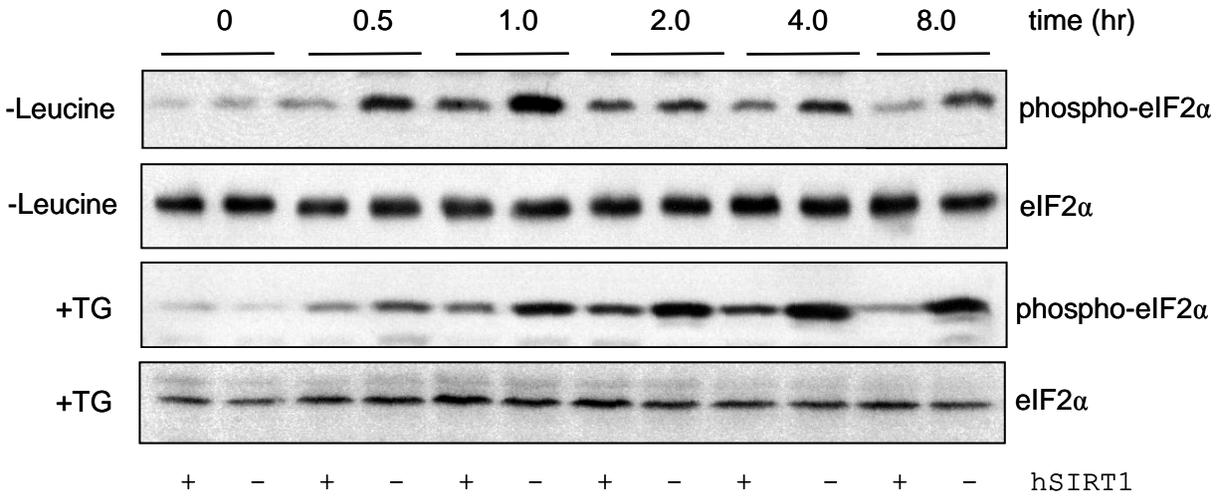


Figure 3.8 SIRT1 depleted HeLa cells show persistently higher level of eIF2 α phosphorylation in response to amino acid starvation and ER stress

SIRT1 depleted and matched control cells were treated with mock treatment, leucine starvation or thapsigargin (ER stress) for various time periods as indicated. Whole cell lysates were analyzed by western blot analysis using phospho- or total protein antibody for eIF2 α . +: Negative control RNAi HeLa cells, -: SIRT1 RNAi HeLa cells.

We further confirmed this observation in SIRT1 knock-out mouse embryonic fibroblasts. Consistent with our data from HeLa cells, the SIRT1 depleted MEFs showed higher level of eIF2 α phosphorylation which was more sustained in response to ER stress (Figure 3.9).

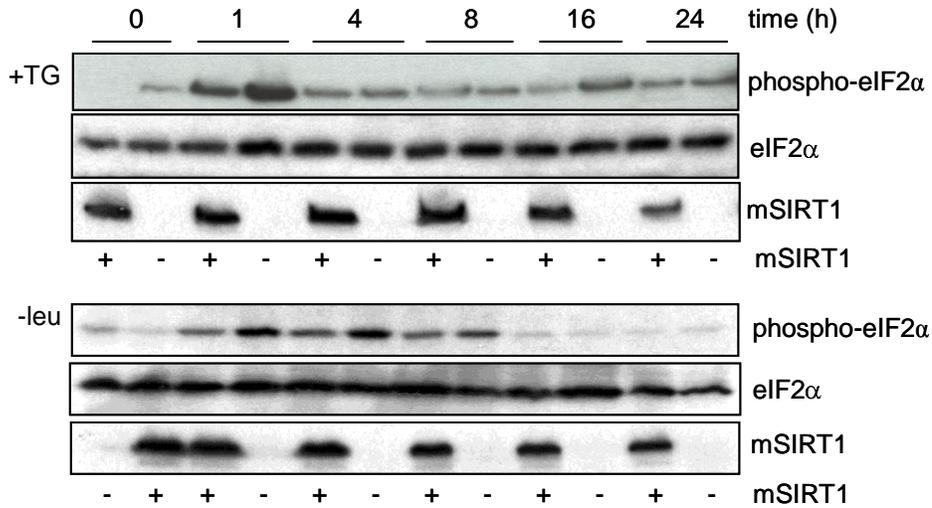


Figure 3.9 SIRT1 deficient mouse embryonic fibroblasts show higher level of eIF2 α phosphorylation in response to amino acid starvation and ER stress

SIRT1 knock-out and WT MEFs were treated with mock treatment, leucine starvation or thapsigargin (ER stress) for various time periods as indicated. Whole cell lysates were analyzed by western blot analysis using phospho- or total protein antibody for eIF2 α . +: SIRT1^{+/+} MEFs, -: SIRT1^{-/-} MEFs.

3.4.5 SIRT1 deficient cells show delayed CHOP expression in response to cellular stress

Downstream to phosphorylation of eIF2 α in response to cellular stress is the expression of stress response proteins such as ATF4 and CHOP. Investigating the downstream effect of eIF2 α phosphorylation in WT versus SIRT1 knock out cells, we found that the SIRT1 deficient cells, which demonstrated higher eIF2 α phosphorylation, surprisingly showed a much delayed expression of the stress response protein CHOP (Figure 3.10). In addition we also observed reduced levels of CyclinD1 in SIRT1 deficient cells, which can be explained by translation attenuation of global protein synthesis in response to sustained eIF2 α phosphorylation in absence of SIRT1.

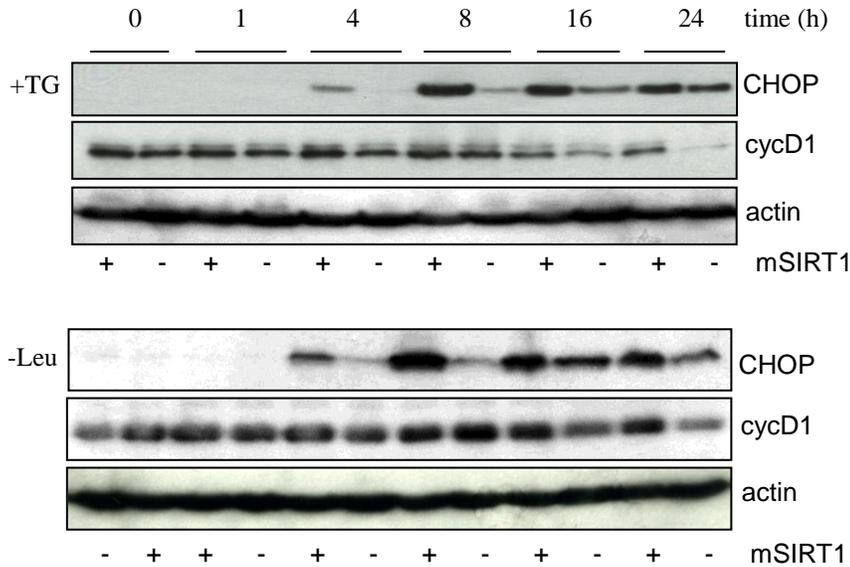


Figure 3.10 SIRT1 deficient mouse embryonic fibroblasts show delayed CHOP expression response to ER stress and amino acid starvation.

SIRT1 knock-out and WT MEFs were treated with mock treatment, leucine starvation or thapsigargin (ER stress) for various time periods as indicated. Whole cell lysates were analyzed by western blot analysis using phospho- or total protein antibody for eIF2 α . +: SIRT1^{+/+} MEFs, -: SIRT1^{-/-} MEFs.

Downstream to CHOP is GADD34 which acts in a feedback loop to dephosphorylate eIF2 α with the help of protein phosphatase1 (PP1). Thus, the explanation of higher and more sustained eIF2 α phosphorylation in SIRT1 deficient cells could be rather due to impaired dephosphorylation of eIF2 α due to delayed expression of CHOP and possibly its downstream effectors.

3.4.6 SIRT1 deficient cells are more sensitive to free radical stress

eIF2 α phosphorylation is critical to cell survival under various stress conditions including oxidative stress. The later part of stress response involves feed back inhibition of eIF2 α

phosphorylation by its downstream target CHOP and GADD34, which binds the eukaryotic serine/threonine phosphatase protein phosphatase 1 (PP1) to direct eIF-2 α dephosphorylation, thereby recovering translation to aid expression of stress proteins and help cells recover from stress [35,59,225]. Thus, delayed expression downstream targets of the stress response pathway can be detrimental to cell survival. Since, our results suggested that SIRT1 depleted cells have delayed expression of CHOP and persistently higher levels of eIF2 α phosphorylation, we asked if SIRT1 null cells were more sensitive to stress. To investigate this, we treated SIRT1 null and wild-type mouse embryonic fibroblasts (MEFs) for 24 hrs with increasing dose of oxidative stress causing agents, paraquat and H₂O₂. Our results showed that SIRT1 null MEFs were significantly more sensitive to oxidative stress compared to wild-type MEFs (Figure 3.11). This observation was consistent with the slower stress response and sustained upregulated eIF2 α phosphorylation in SIRT1 depleted cells.

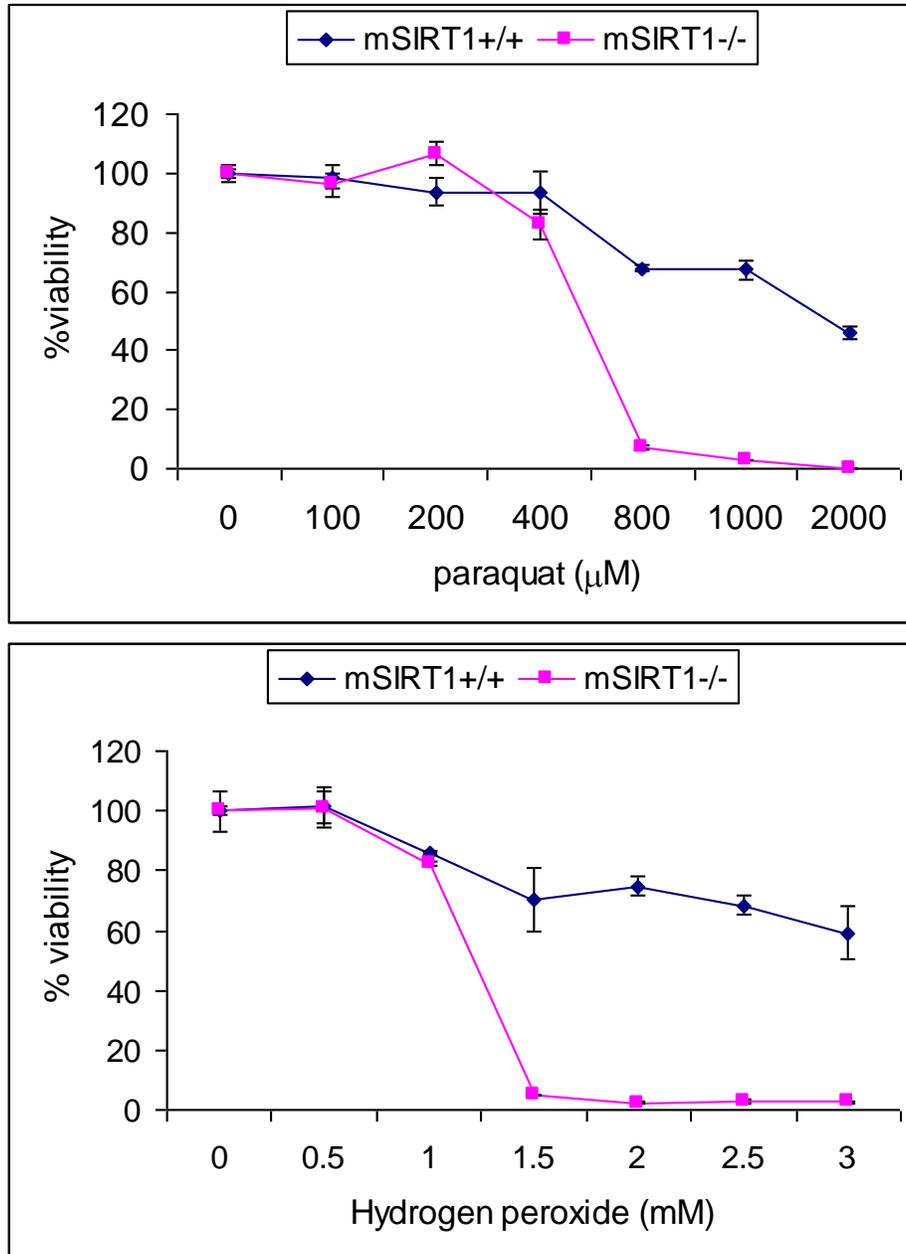


Figure 3.11 SIRT1 null mouse embryonic fibroblasts are more sensitive to oxidative stress

Wild-type and SIRT1 null mouse embryonic fibroblasts were subjected to indicated doses of paraquat and hydrogen peroxide for 24 hr. MTT assay was used to detect cell survival. Data is presented as percentage survival with regards to mock (vehicle alone) treatment. Bars = \pm SEM

3.4.7 Increased sensitivity of SIRT1 deficient cells is caused by translation inhibition

To further examine if the increased sensitivity of SIRT1 depleted cells was because of the potential block in translation due to sustained eIF2 α phosphorylation, we treated the control and SIRT1 depleted HeLa cells with a translation inhibitory drug, blasticidine, while they were subjected to hydrogen peroxide treatment and assayed cell survival.

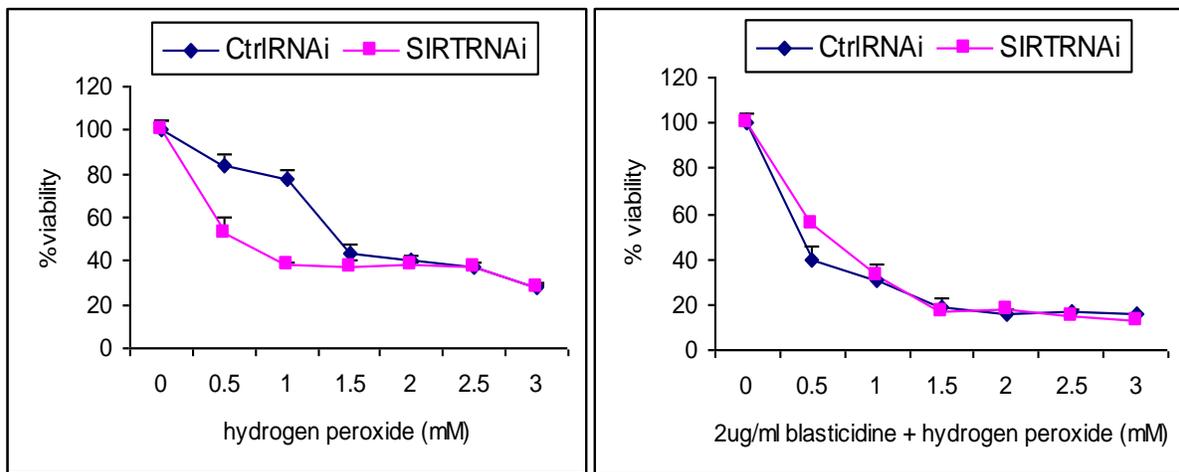


Figure 3.12 Translation inhibition leads to increased sensitivity of SIRT1 positive cells to oxidative stress.

SIRT1 depleted and matched control HeLa cells were treated with increasing dose of hydrogen peroxide as indicated, with or without 2 μ g/ml blasticidine for 24 hrs. MTT assay was used to detect cell survival. Data is presented as percentage survival with regards to mock (vehicle alone) treatment.

As shown in Figure 3.12, our results showed that the SIRT1 positive cells become similarly sensitive to oxidative stress as SIRT1 depleted cells when translation was blocked. This result further suggests that the increased sensitivity of SIRT1 depleted cells to oxidative stress could be potentially a result of impaired translation recovery due to sustained eIF2 α phosphorylation.

3.4.8 SIRT1 deficient cells show impaired translation recovery in response to ER stress

To investigate whether higher eIF2 α phosphorylation and more sensitivity toward cellular stress in SIRT1 knock-out cells was indeed due to impaired translation recovery, we examined new protein synthesis in ER stressed cells by ^{35}S -Methionine incorporation assay. As shown in Figure 3.13, recovery of protein synthesis in SIRT1 deficient cells in response to ER stress was slower than in WT MEFs.

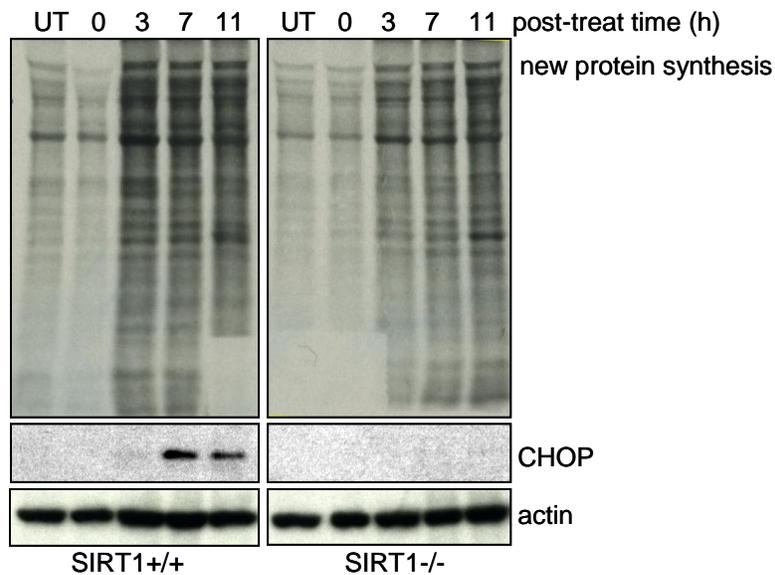


Figure 3.13 SIRT1 null mouse embryonic fibroblasts show slower translation recovery in response to ER stress

Wild-type and SIRT1 null mouse embryonic fibroblasts were treated with 2 μM thapsigargin for 1hr. Cells were then pulsed with 200 $\mu\text{Ci/ml}$ ^{35}S labeled methionine for 30 mins at each time point. Proteins extracts from cells were analysed by western blot analysis. New protein synthesis was assessed by incorporation of ^{35}S in cellular protein detected by autoradiography of the protein blots. Expression of CHOP was detected by probing the blot with anti-CHOP antibody. Actin was used to detect equal protein load in all lanes.

3.4.9 SIRT1 and eIF2 α associate regardless of stress condition

Since SIRT1 depleted cells showed higher eIF2 α phosphorylation under stressed conditions and downstream stress response, we wished to investigate if SIRT1-eIF2 α association is regulated by stress environment. HeLa cells were transfected with flag-tagged SIRT1 expression plasmid and myc-tagged eIF2 α expression plasmid, and treated with -leucine or tunicamycin. Whole cell extracts were examined for SIRT1-eIF2 α association by co-immunoprecipitation using epitope tag antibodies. The results demonstrated that SIRT1 associated with eIF2 α regardless of stress condition (Figure 3.14).

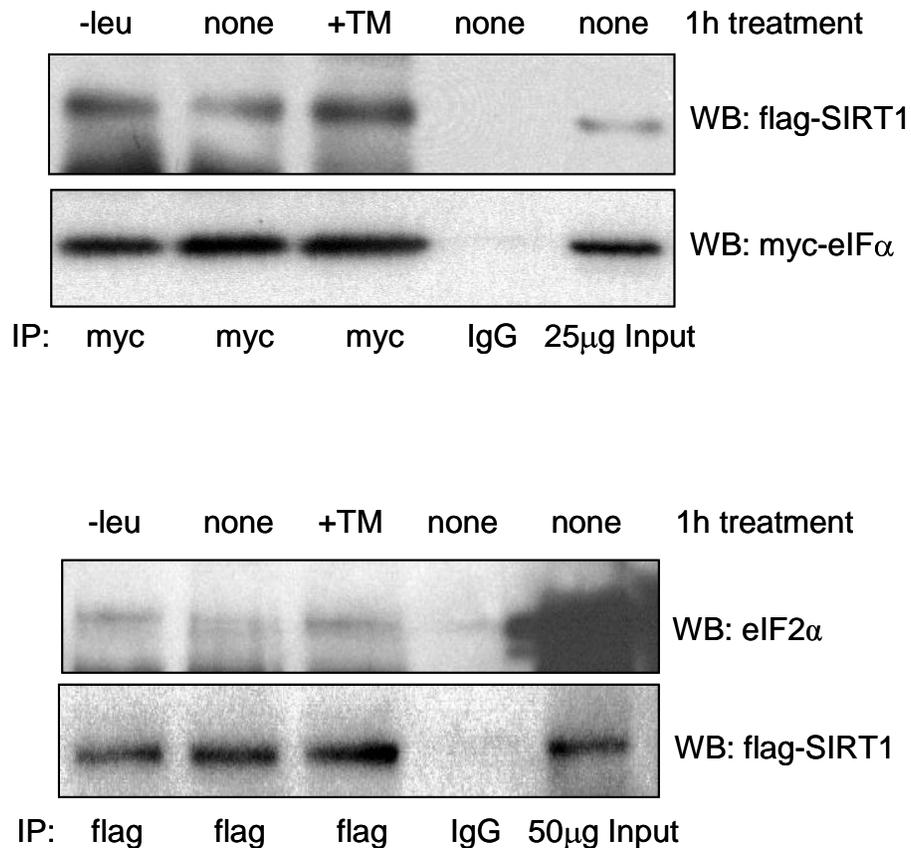


Figure 3.14 SIRT1 associates with eIF2 α regardless of stress condition

HeLa cells were transfected with flag-tagged SIRT1 and myc-tagged eIF2 α expression plasmids. 24h post-transfection, cells were either mock-treated or treated for with –leucine media or tunicamycin (2 μ g/ml) for 1 hr. Whole cell lysates were used for immunoprecipitation of SIRT1 or eIF2 α using anti-flag-tag or anti-myc-tag antibody respectively and western blotted for eIF2 α or flag antibody for SIRT1. IgG antibody was used as negative control of immunoprecipitation and 25 or 50 μ g whole cell lysate was used as input. The blots were also probed for the immunoprecipitated proteins.

3.4.10 SIRT1 catalytic activity or eIF2 α phosphorylation does not affect SIRT1-eIF2 α association.

SIRT1 is an NAD⁺ dependent deacetylase whose catalytic activity has been shown to play a role in most of its cellular functions. So, we wished to examine if the catalytic domain of SIRT1 played any role in SIRT1-eIF2 α association. For this, we used the catalytic mutant of SIRT1, SIRT1-H363Y, in which the catalytic core domain was mutated by substituting the critical histidine-363 residue with a tyrosine. HeLa cells were transfected with flag-tagged SIRT1, flag-tagged SIRT1H363Y, myc-tagged eIF2 α or myc-tagged eIF2 α S51A (phosphorylation mutant eIF2 α) as indicated, and SIRT1-eIF2 α association was examined by co-immunoprecipitation. As shown in Figure 3.15 our result suggests that wild-type as well as the catalytic mutant SIRT1 interacts with eIF2 α . Furthermore, wild-type as well as the phosphorylation mutant eIF2 α , both associated with SIRT1 in HeLa cells.

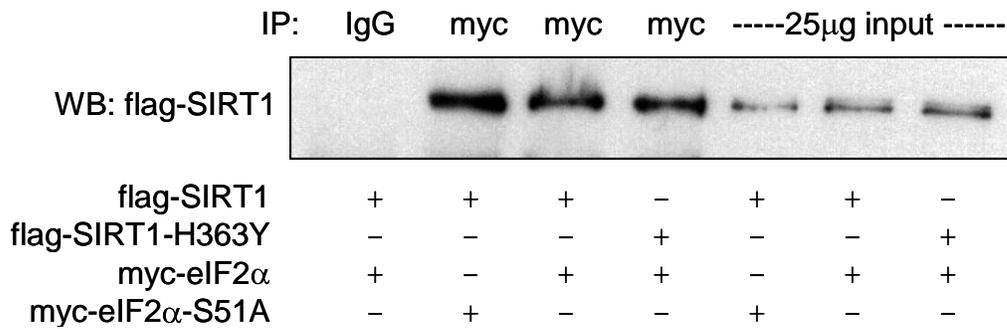


Figure 3.15 SIRT1 associates with eIF2α regardless of SIRT1's catalytic activity or eIF2α's phosphorylation status

HeLa cells were transfected with the indicated expression plasmids. 24h post-transfection whole cell lysates were extracted and used for immunoprecipitation of SIRT1 or eIF2α using anti-flag-tag or anti-myc-tag antibody respectively and western blotted with flag antibody for SIRT1. IgG antibody was used as negative control of immunoprecipitation and 25µg whole cell lysate was used as input.

Next we asked if SIRT1 may bind phosphorylated eIF2α more preferentially than un-phosphorylated eIF2α. For this, we over-expressed SIRT1 either with the phosphorylation mutant eIF2α, eIF2αS51A, or with the phosphorylation mimetic eIF2α, eIF2αS51D in HeLa cells and analyzed SIRT1-eIF2α interaction by co-immunoprecipitation. As shown in figure 3.16, our results further confirmed that the association of SIRT1 and eIF2α is not dependent on the phosphorylation state of eIF2α as the phosphorylation mutant and the phosphorylation mimetic eIFα, both interacted with SIRT1.

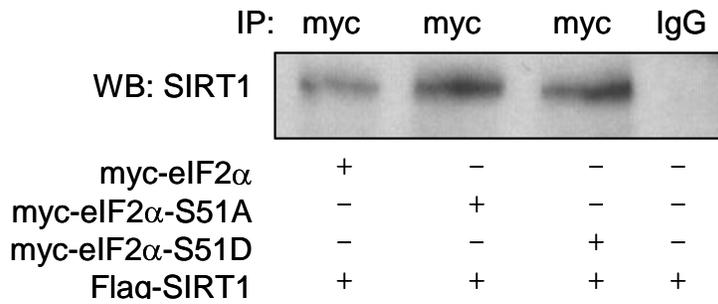


Figure 3.16 SIRT1 associates with the phosphorylation mutant and mimetic eIF2 α

HeLa cells were transfected with the indicated expression plasmids. 24h post-transfection whole cell lysates were extracted and used for immunoprecipitation of eIF2 α using anti-myc-tag antibody. Extracts were then analysed by western blotting using flag antibody for SIRT1. IgG antibody was used as negative control of immunoprecipitation.

We also attempted to examine if eIF2 α is an acetylated protein by immunoprecipitating eIF2 α under stressed or unstressed conditions and probing with anti-acetyl lysine antibody. However, we could not detect acetylation of eIF2 α in our experiment (data not shown).

3.5 DISCUSSION

Regulation of gene expression at the post-transcriptional and translational level is an important control mechanism. Translation is the final step in the flow of genetic information and regulation at this level provides the cells with the capability to respond rapidly to changes in physiological condition in response to environmental changes. Conditions such as cellular stress due to heat, irradiation, hypoxia, nutrient deprivation, or apoptosis require immediate changes in the protein levels of cells. Hence, cells have evolved a unique mechanism by which they can quickly modulate the process of translation as per requirement. Under stressful conditions, rather than investing energy in making more protein for maintenance, growth and proliferation (which consumes up to an estimated 50% depending on the type of organism, [260,309]), cells use their energy for cell survival processes. Attenuation of translation not only reduces the load of protein synthesis on cells under stress, but also prevents synthesis of unwanted proteins that may interfere with cellular stress response. Remarkably, however, despite the stress induced translation attenuation, selective translation of stress responsive proteins is induced, indeed by the same mechanism. eIF2 α , a eukaryotic translation initiation factor, is a critical regulator for translation in response to cellular stress. eIF2 α phosphorylation by specific kinases in response to specific stress stimuli induces attenuation of global translation, accompanied by selective translation of proteins that are required for cell survival under stress.

In an effort to identify novel binding partners for SIRT1, we discovered that SIRT1 interacts with eIF2 α . This was interesting because, while eIF2 α plays a critical role in regulating cellular stress response by attenuating translation under stress conditions, SIRT1 is believed to regulate cell cycle, cellular senescence, apoptosis and metabolism, by functional interactions

with a number of transcription factors and other proteins. Calorie restriction mediated lifespan extension in yeast and worms have been shown to be dependent on SIRT1. Apart from regulating metabolism, SIRT1 is also believed to delay the aging process by regulating cellular stress response such as oxidative stress and DNA damage. SIRT1 protects against genotoxic stress and senescence by deacetylating and inactivating p53 [49,178]. It prevents stress-induced premature senescence and inhibits apoptosis by regulating p53, E2F, FOXO and Ku70 [55,231,232,281,298,305]. Stress resistance is often considered as an effector of longevity. SIRT1's widespread role in protection against stress indicates a potential link between SIRT1 mediated stress resistance and longevity. Thus, SIRT1's association with eIF2 α was very interesting, because it indicated a potential novel role of SIRT1 in regulating stress response through translation control.

Interestingly, although SIRT1 is predominantly known to be a nuclear protein, our data (figure 3.5 and 3.6) indicated that SIRT1 localizes in the cytoplasm of multiple mammalian cell types such as HeLa, 293T, Jurkat and murine embryonic fibroblasts. Others have reported various cytoplasmic substrates and functions of SIRT1, such as IRS2 [326] and acetyl-CoA [110] and SIRT1's role in inhibiting Bax mediated apoptosis via Ku70. The cytoplasmic localization and functions of SIRT1 are very relevant to our observation that SIRT1 associates with eIF2 α since the major function of eIF2 α is in protein synthesis, which is a cytoplasmic process. Interestingly, the majority of SIRT1 substrates identified thus far are transcription factors, which mediate SIRT1's role by regulating gene expression at the level of transcription. Thus, SIRT1's association with eIF2 α suggested a novel category of SIRT1 target proteins, namely a 'translation' factor, thereby indicating the potential role for SIRT1 in translational regulation.

Phosphorylation of eIF2 α by four different eIF2 α kinases in response to specific stress stimuli is one of the early steps in the coordinated cellular stress response. While under stress, cells have to reprogram their gene expression such that it can reduce the load of protein synthesis to save energy and at the same time make sure that translation of proteins required for cell survival during stress conditions is activated. The integrated stress response involves 1) inhibition of growth and proliferation by translation attenuation of global proteins, 2) increased expression of chaperone proteins to increase cellular protein folding capacity for maintenance of macromolecular integrity, 3) increased synthesis of repair and damage control proteins such as glutathione and superoxide-dismutase to recover from stress related damage and finally 4) induction of apoptotic pathway proteins in case of severe damage where eliminating damaged cells is important to protect the organism [169]. eIF2 α plays a role in the rate limiting step of protein synthesis, and phosphorylation of eIF2 α is cardinal to translation attenuation in response to cellular stress and induction of stress protein synthesis.

Since SIRT1 also plays a role in stress response, we investigated if its association with eIF2 α has any relevance to stress-mediated eIF2 α phosphorylation. Interestingly, we observed that depletion of SIRT1 resulted in increased eIF2 α phosphorylation in response to various stress stimuli. In order to verify whether or not the difference in phosphorylation of eIF2 α between SIRT1 depleted and control cells was a time dependent effect, we further performed a time course for ER stress (thapsigargin) or a.a starvation (-leucine) in wild-type and SIRT1 depleted cells. We found that, eIF2 α phosphorylation was much more robust and sustained in SIRT1 depleted cells. Notably, we saw faster and more prominent induction of eIF2 α phosphorylation in SIRT1 depleted cells in response to both stress types; a.a starvation and TG induced ER stress.

Interestingly though, we observed that the SIRT1 deficient cells which showed higher eIF2 α phosphorylation showed much delayed expression of the stress responsive protein CHOP (Figure 3.10). One of the important regulatory mechanisms of eIF2 α mediated stress response is the negative feedback loop that functions later in the stress response and plays a role in translation recovery after the initial stress induced attenuation of protein synthesis. This process involves dephosphorylation of eIF2 α by proteins that are synthesized downstream to the stress response signaling cascade. The feed-back inhibition of eIF2 α phosphorylation is important for translation of genes required for recovery and survival of stressed cells. A delayed expression of the downstream stress response proteins can hinder with the dephosphorylation of eIF2 α through the negative feedback loop, which in turn can affect translation recovery in the later phase of stress response.

Since we observed that SIRT1 depleted cells demonstrate delayed CHOP expression and a more sustained eIF2 α phosphorylation in response to stress, we investigated if SIRT1 deficient cells were more sensitive to stress. In response to a 24 hour-long free radical stress induced by two different agents; paraquat and hydrogen peroxide, the SIRT1 null cells showed significantly more sensitivity compared to the matched control cells. This suggests that SIRT1 may be important for cell survival during the prolonged stress response. This is consistent with our observation that SIRT1 deficient cells show sustained eIF2 α phosphorylation which could be due to impaired translation recovery during the later phase of stress response, thereby rendering cells more prone to death.

We attempted to investigate this further by examining new protein synthesis in SIRT1 deficient versus wild-type cells at various time points post-stress treatment by 35S-radiolabeled

methionine incorporation. Our results show that translation recovery post stress treatment is indeed slower in SIRT1 deficient cells compared to wild-type cells. While our results suggest a correlation between delayed CHOP expression and sustained eIF2 α phosphorylation in SIRT1 deficient cells to their slower translation recovery and increased sensitivity to stress, further investigation will be required to elucidate whether the increased sensitivity of SIRT1 null cells due to impaired GADD34/PP1 activity leading to defective recovery of translation in late phase of stress response.

SIRT1 targets numerous stress/survival factors, such as, p53, FOXO, p300, Ku70, E2F and p73, regulating them to ultimately favor protection under stress conditions. While on one hand SIRT1 deacetylates and downregulates FOXO1, 3, and 4 thereby repressing FOXO-mediated apoptosis [34,216], on the other hand, certain FOXO targets, such as the DNA repair gene GADD45, are activated by SIRT1 [34,63,298]. SIRT1 protects against β -amyloid induced death in microglia and neural toxicity in *C.elegans*, potentially by down-regulating p53 and FOXO. It also protects pancreatic beta cells against oxidative stress by activating FOXO1 [108]. Thus, SIRT1 has been documented to play a pro-survival role under stress by regulating transcription. Our results, however, indicate a potential novel regulatory mechanism; whereby, SIRT1 may play a role in cellular stress response by regulating translation via eIF2 α phosphorylation.

Since our data indicated that SIRT1 depletion leads to increased phosphorylation of eIF2 α under various stress conditions, we suspected that SIRT1's association to eIF2 α may be regulated by stress conditions. Surprisingly, we did not observe any significant difference in SIRT1-eIF2 α association with regards to unstressed or stressed conditions. Also, since SIRT1

functions predominantly by deacetylating various target proteins, we examined if the association of SIRT1 with eIF2 α depended on the integrity of SIRT1's catalytic domain. Interestingly, we observed that SIRT1 associates with eIF2 α regardless of an intact catalytic domain. Furthermore, we could not detect eIF2 α to be acetylated under stressed or unstressed conditions. By examining the phosphorylation of eIF2 α kinases in SIRT1 depleted cells in response to stress, we could not detect any difference in the activity of these kinases with regards to SIRT1's presence or absence (data not shown). Although we can not rule out the regulation of eIF2 α phosphorylation by deacetylase activity of SIRT1, it is also possible that SIRT1 regulates eIF2 α phosphorylation by acting as a scaffold protein, thereby binding and masking the potential phosphorylation site on eIF2 α . In this regard, we investigated if SIRT1 had any binding preference for phosphorylated versus de-phosphorylated versions of eIF2 α , by using a phosphorylation mutant eIF2 α , eIF2 α S51A and a phosphorylation mimetic eIF2 α , eIF2 α S51D. However, our results showed that SIRT1 binds to eIF2 α , irrespective of its phosphorylation status. While this observation is slightly puzzling, it is possible that when exogenously over-expressed, these two proteins have strong enough affinity for each other to bind regardless of any conditions. Alternatively, there could be a temporal regulation of SIRT1-eIF2 α association with regards to eIF2 α de-phosphorylation.

While the mechanism of SIRT1 mediated regulation of eIF2 α is still unclear, our data suggests a role for SIRT1 in eIF2 α mediated stress response, thereby indicating, for the first time, a role for SIRT1 in translation regulation to control cellular stress response. Given that SIRT1 plays a role in cell survival and protection against age-related diseases, its role in

translation attenuation, in response to cellular stress, can open up new directions for SIRT1 mediated therapeutic intervention of stress related diseases.

**4.0 SIRT1 NEGATIVELY REGULATES MAMMALIAN TARGET OF RAPAMYCIN
(TOR) THROUGH TSC2**

Hiyaa Singhee Ghosh *et al.*

4.1 ABSTRACT

Calorie restriction (CR) or mutations in Insulin/insulin-like growth factor (IGF)-1 signaling are two conserved pathways implicated in longevity in yeast, worms, flies and mice. SIRT1 is required for CR mediated lifespan extension in yeast, worm, fly and potentially in mice. SIRT1 is also believed to be involved in insulin/IGF-1 signaling mediated longevity in a range of organisms by acting through the forkhead proteins. Downstream to the insulin/IGF-1 pathway is the target of rapamycin (TOR), which has been implicated in regulating cell growth and stress resistance in response to nutrients, growth factors, energy and stress conditions. TOR has been found to be involved in extending lifespan in yeast and *Drosophila*. The mammalian target of rapamycin (mTOR) and SIRT1, have both been shown to be involved in several age-related diseases. Although the mechanism by which SIRT1 potentially regulates longevity in mammals is still not understood, it is conceivable that SIRT1 may regulate the IGF-1/mTOR signaling to mediate longevity in mammals.

Here we demonstrate that SIRT1 regulates mTOR signaling. SIRT1 deficient or depleted cells show elevated mTOR activity. In addition, SIRT1 activator, resveratrol reduces whereas, inhibitor, nicotinamide, enhances mTOR activity in a SIRT1 and TSC2-dependent manner. Furthermore, SIRT1 physically associates with TSC2 *in vivo*. These results indicate that SIRT1 potentially regulates mTOR signaling through its deacetylase activity on the TSC1/TSC2 complex. Thus, we demonstrate for the first time that SIRT1 and mTOR, two proteins that are involved in longevity pathways and age related diseases, are potentially interconnected at a point upstream of the mTOR complex-1.

4.2 INTRODUCTION

In the past few decades, work in the field of aging research has indicated that aging, like most physiological functions, is a regulated process mediated through multiple signaling pathways. Common genetic and environmental factors regulate aging in different species through diverse regulatory mechanisms showing that some of the pathways believed to be involved in aging are evolutionarily conserved. Caloric restriction (CR) and the Insulin/IGF-1 signaling pathway are two such conserved mechanisms that have been shown to regulate lifespan in a wide range of organisms: from the unicellular yeast to mammals.

SIRT1, the NAD⁺ protein deacetylase has been shown to extend lifespan in yeast, worms, flies and potentially in mammals. Interestingly, CR has been shown to increase lifespan in yeast, *Drosophila*, *C. elegans* and mice [104,146,165,256,257,277,311,313], and reduces many age related physiological symptoms in mammals such as free radicals, damaged macromolecules, plasma insulin, body temperature, and weight. CR has been shown to also delay the onset of many age related diseases in mammals such as cancer, diabetes and various neurodegenerative disorders [168]. Furthermore, the CR mediated lifespan extension in yeasts, worms and flies have been shown to be SIRT1 dependent. Sirtuins are known to mediate at least some of the effects of CR and extend lifespan, potentially by regulating stress response and metabolism [41].

Genetic analyses of the aging processes in multi-cellular organisms, including *C. elegans*, *Drosophila* and mice have shown that a single-gene-mutation significantly extends lifespan in all these organisms. Like CR, modulation of genes in the insulin-signaling pathway, which alters nutrient sensing, has been shown to extend lifespan in various species [103,104,203,238,291]. It has been theorized that CR regulates certain genetic pathways that shift an organism's metabolic

investment from reproduction and growth toward somatic maintenance, allowing survival under stressed conditions until there are suitable reproductive conditions [124,161,291]. The best characterized pathway so far is the insulin/insulin-like growth factor-1 endocrine system. In *C. elegans*, in response to starvation or crowding, the insulin/IGF-1 signaling pathway controls the formation of the dauer larva, an alternative developmental state that is non-reproducing, stress resistant, and long lived [164,254]. Mutations in genes of the same pathway, *daf-2* (insulin receptor) and *age-1* (phosphatidyl inositol-3 kinase), lead to doubling of lifespan, and render the animals more sensitive to dauer formation [157]. Both the dauer formation and lifespan-extension phenotypes are suppressed by mutations in *daf-16*, a forkhead family transcription factor [157,304]. Similarly, *Drosophila* mutations in *Inr* (insulin-like-receptor) and *chico* (insulin-receptor substrate), both regulate lifespan [54,291]. The insulin/IGF-1 signaling pathway is nutrient activated and decreased signaling through this pathway increases lifespan in *C. elegans* and mice [156,157]. Interestingly, the SIRT1 orthologs have been shown to interact with insulin/IGF-1 signaling pathway in *C. elegans* and mice. In particular, SIRT1 regulates the IGF-1/Akt/FOXO signaling pathway in *C. elegans*, and mice through deacetylation of FOXO [34,55,294]. Also, CR has been shown to reduce rodent serum IGF-1 levels [72] and this reduction appears to mediate, at least in part, its lifespan prolonging effects [168,275,281], thus linking the reduction in IGF-1 mediated longevity to CR. Interestingly, Cohen et al. [55] showed that CR-induced SIRT1 induction could be attenuated by IGF-1 and treatment of cells with either insulin or IGF-1 lowers SIRT1 levels, indicating the inverse relationship between SIRT1 and insulin/IGF-1 pathway in stress resistance, cell survival and longevity.

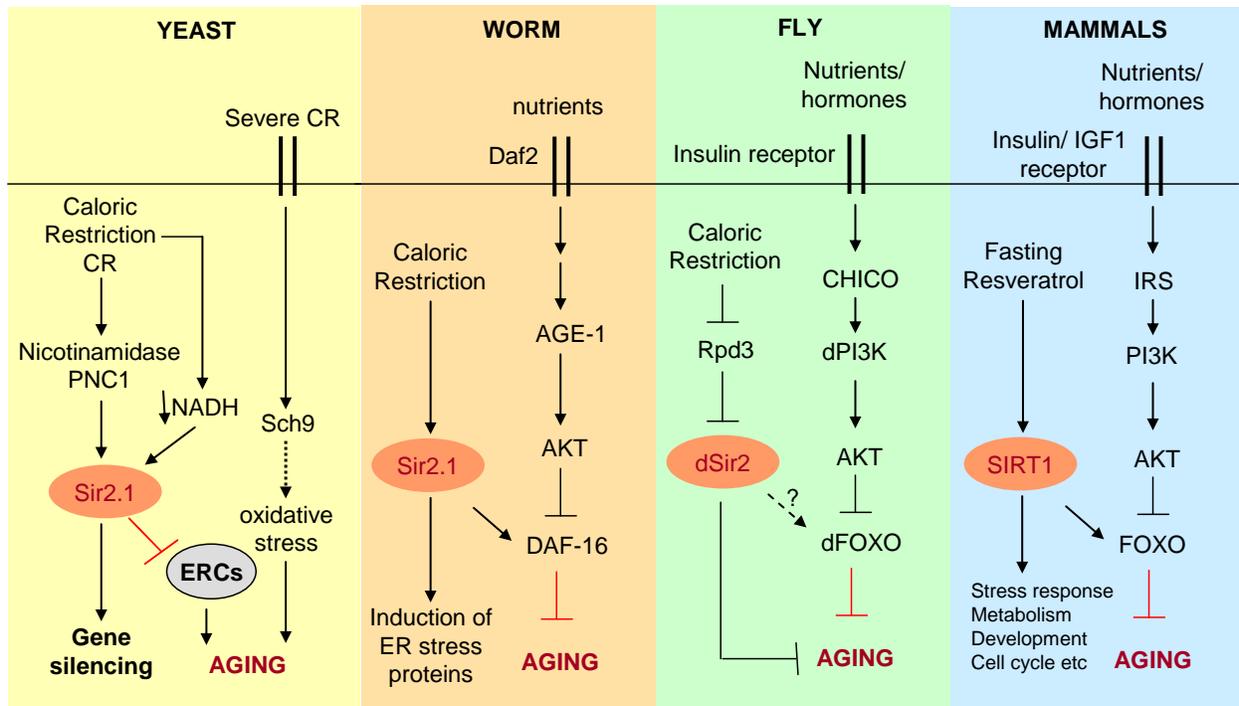


Figure 4.1 SIRT1, CR and IGF-1 signaling in aging and longevity

In yeast Sir2p (SIRT1) activity is increased through increased expression of the NAD⁺ salvage pathway enzyme, nicotinamidase PNC1, but also by increasing the NAD⁺/NADH ratio (or lowering NADH levels) in response to calorie restriction (CR). Main cause of aging in yeast is considered to be the exponential accumulation of extrachromosomal circular DNA repeats (ERCs). Increased NAD⁺ and PNC1 which activates Sir2p, inhibit the formation of the deleterious extrachromosomal circular DNA repeats (ERCs) and contribute to life span extension in yeast. The glucose and nutrients fermentation pathway activates the AKT-related kinase Sch9 which induces oxidative stress and participates in yeast aging. Under severe CR inactivation of this pathway promotes longevity in yeast. The longevity pathway is conserved amongst worms, flies and mammals. Activation of the insulin growth factor receptor IGFR (Daf-2/dIGFR) activates the insulin receptor substrate IRS (p65/CHICO), which stimulates phosphoinositol-3 kinase PI3K (AGE-1/dPI3K), which in turn phosphorylates the PKB/AKT kinase. AKT phosphorylates and inactivates FOXO (Daf-16/dFOXO). Inactivation of the insulin receptor pathway promotes longevity in worms, drosophila, and mammals through increased activity of FOXO. Sir2/SIRT1 activation induces life span extension through interaction with Daf-16/FOXO factors. PNC1=pyrazinamidase/Nicotinamidase 1; Sir2p=yeast silent information regulator 2; NADH=reduced form of nicotinamide adenine dinucleotide; Sch9=Saccharomyces cerevisiae protein kinase 9; Daf-16='dauer' larvae transcription

factor-16; AGE-1=worm homolog of the phosphoinositol-3 kinase; AKT=protein kinase B; Rpd3=reduced potassium dependence 3 (class II histone deacetylase); IGF-1=insulin growth factor-1; FOXO=forkhead box subgroup 'O' transcription factor; IRS=insulin receptor substrate; CHICO=drosophila homolog of IRS; SIRT1=sirtuin 1.

In *Drosophila*, nutrient and insulin signaling have been shown to be critical factors in regulating not only lifespan but also growth and size [230]. Mutations of genes involved in the insulin signaling pathway have shown reduction in fly size [25,32,180]. The highly conserved TOR (target of rapamycin) kinases, which is a downstream component of the insulin/IGF-1 signaling pathway, has emerged as a prominent player in regulating cell growth, size and longevity. Recent evidence suggests that the fat body in *Drosophila* acts as a nutrient sensor, which uses TOR signaling to generate a humoral signal that modulates insulin signaling and growth in peripheral tissues [57]. Over-expression of dTSC1 or dTSC2, upstream effectors of TOR, or mutation in dTOR or dS6K (downstream TOR substrate), leads to longevity phenotype in *Drosophila* [151]. TOR deficiency in the nematode *C.elegans* was recently shown to extend lifespan [303]. Furthermore, in yeast 6 out of 10 gene mutations that increased the replicative life span corresponded to components of the TOR pathway including TOR and S6K1 (Sch9) that play a role in the regulation of translation [150].

TOR, which is specifically inhibited by the macrolide rapamycin, belongs to a family of phosphatidylinositol kinase-related kinases (PIKK). These proteins contain a carboxy-terminal serine/threonine protein kinase domain that resembles the catalytic domain of phosphatidylinositol 3-kinases (PI3Ks) and PI4Ks. Amino-terminal to the kinase domain in TOR is the FKBP12-rapamycin binding domain. Rapamycin forms a complex with the intracellular cofactor, the peptidyl-propyl *cis/trans* isomerase, FKBP12, which then binds and

inhibits TOR. Single amino acid substitutions in this domain yield TOR proteins (TOR1-1 and TOR2-1) that are no longer bound and inhibited by FKBP12-rapamycin complex. TOR was originally identified by mutations, *TOR1-1*, and *TOR2-1*, that conferred resistance to the growth inhibitory properties of rapamycin, in budding yeast *Saccharomyces cerevesiae* [120]. Subsequent biochemical studies in mammalian cells led to the identification of the mammalian target of rapamycin, mTOR. TOR has been found to be conserved in all eukaryote genomes examined, including yeast, algae, slime mold, plants, worms, flies and mammals. Unlike yeast, which in some cases have two TOR genes, higher eukaryotes possess only a single TOR gene [60,61,179].

Genetic and biochemical studies in yeast have identified that there are two distinct TOR complexes; TOR complex-1 (TORC1) and TOR complex-2 (TORC2) (Figure 4.2). While TORC1 is rapamycin sensitive and regulates the temporal cell growth, TORC2 is rapamycin insensitive and decides the spatial aspects of cell growth.

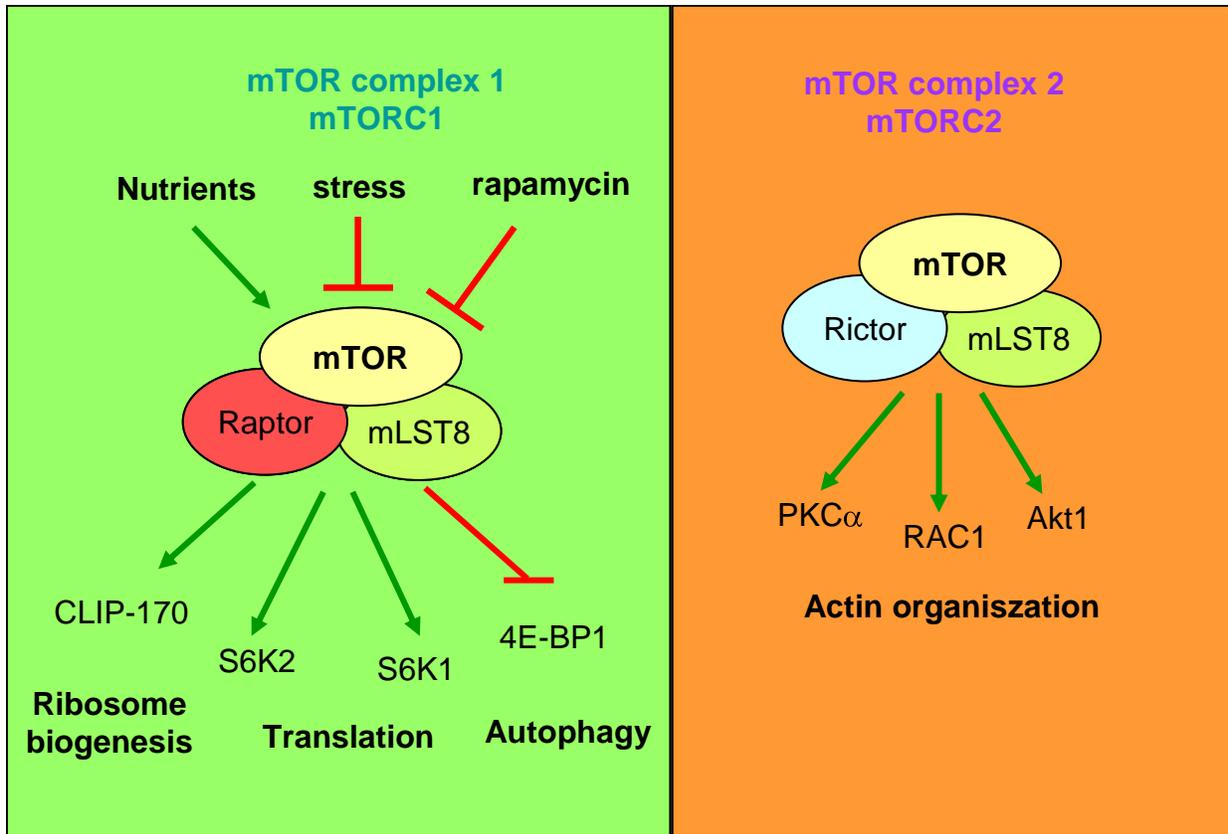


Figure 4.2 TOR complex 1 (TORC1) and TOR complex 2 (TORC2)

The mammalian target of rapamycin (mTOR) exists in two multimeric complexes: mTOR complex 1 (TORC1) and mTOR complex2 (mTORC2). TORC1 mediate the rapamycin-sensitive signaling branch that couples growth cues to the accumulation of mass in response to positive stimuli. TORC2 signaling is rapamycin –insensitive and is required for the organization of the actin cytoskeleton.

Similar to yeast, mammals also have two TOR complexes. mTOR complex-1 (mTORC1) consists of the mTOR (289kD), raptor(150kD) and mLST8 (also called GβL) (36kD), and potentially regulates the temporal aspects of cell growth. mTORC1 is bound by FKBP12-rapamycin which results in inhibition of mTORC1 kinase activity both *in vivo* and *in vitro* [111,158,263]. Although the mechanism by which FKBP12-rapamycin inhibits mTORC1 is not fully understood, it has been shown that FKBP12-rapamycin complex disassociates raptor-mTOR [158], suggesting that FKBP12-rapamycin blocks access to substrates. In another

experiment it was shown that FKBP12-rapamycin inhibits mTORC1 autophosphorylation, suggesting that FKBP12-rapamycin inhibits the intrinsic mTORC1 kinase activity [138].

TOR complex-2 (mTORC2) consists of mTOR, mLST8 and rictor (200kD). Knockdown of mTOR or rictor (but not raptor) results in loss of both actin polymerization and cell spreading, indicating a role in the spatial aspect of cellular growth. mTORC2 is neither bound by FKBP12-rapamycin nor is inhibited by it [138,263]. TOR kinases integrate various signals to regulate cell growth. The four major inputs that are sensed by mTOR are: growth factors, nutrients, energy and stress (Figure 4.3).

mTOR pathway responds to growth factors via the phosphoinositide-3-kinase (PI3K) pathway. Binding of insulin/IGF-1 to their receptors leads to the recruitment and phosphorylation of the insulin receptor substrate (IRS) and subsequent recruitment of P13K. This activates the PI3K signaling in which PI3K bound to IRS converts phosphatidylinositol-4, 5-phosphate (PIP₂) to phosphatidylinositol-3, 4, 5-phosphate (PIP₃). PIP₃ co-recruits PDK1 and Akt to the membrane, resulting in the phosphorylation and activation of Akt by PDK1. PIP₃ accumulation is antagonized by the lipid phosphatase PTEN. Activation of Akt enables it to phosphorylate and inactivate TSC2.

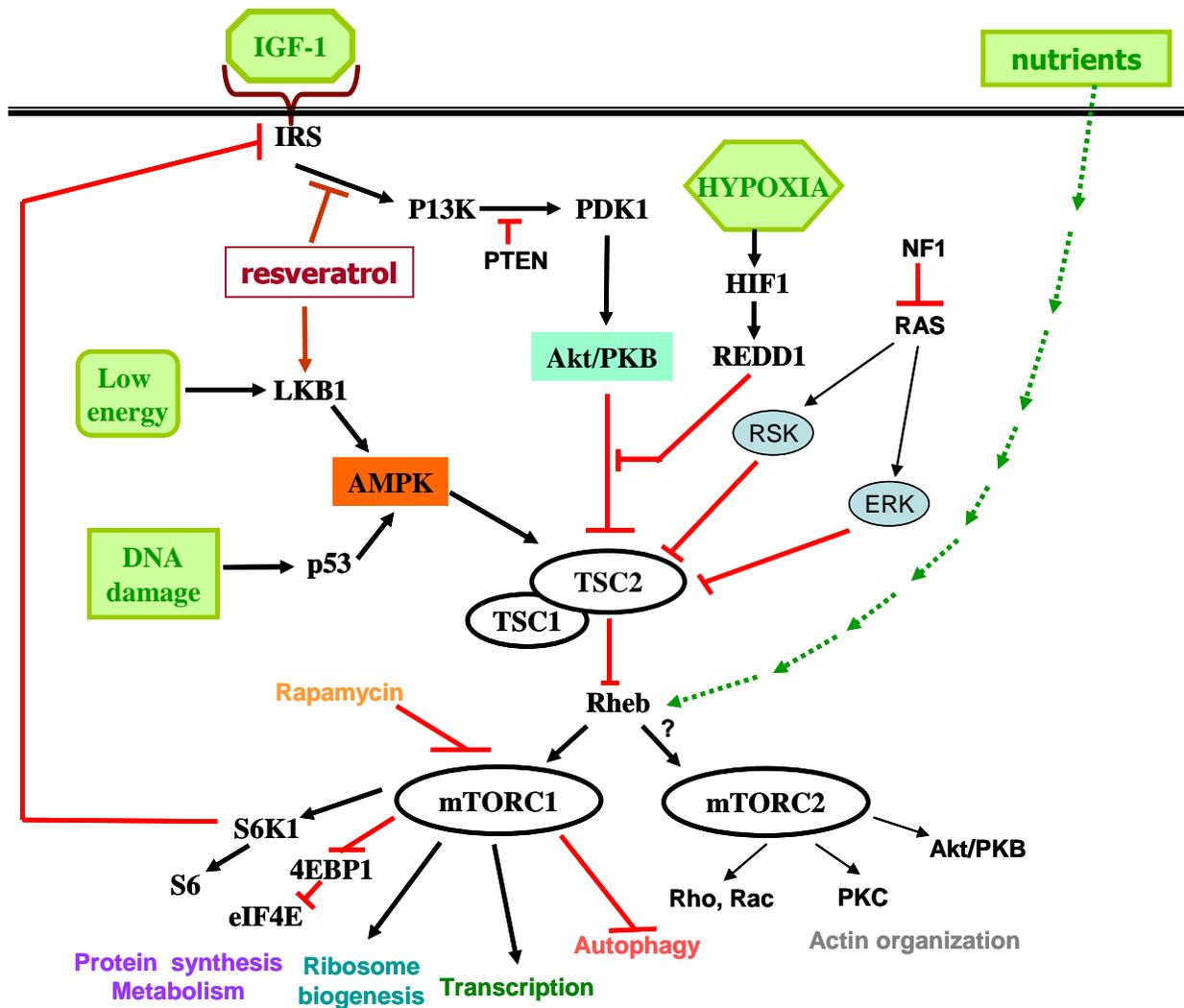


Figure 4.3 mTOR signaling in mammalian cells

In response to growth factors (insulin/IGF-1), energy status, nutrients (amino acids) and stress signals, various signaling pathways are activated. Most of these pathways converge at the major upstream regulator of mTOR, the TSC1/TSC2 complex, which in turn regulate the downstream mTOR activator Rheb.

TSC1-TSC2 acts as a heterodimer that negatively regulates mTOR signaling by acting as a GAP (GTPase activating protein) for small GTPase Rheb [195,196]. Although Rheb binding to mTOR is independent of Rheb possessing the guanyl nucleotide, the GTP bound Rheb is an activator of mTORC1, while the nucleotide-free Rheb acts as an inhibitor [195,196,279].

Inactivation of TSC2 by Akt phosphorylation thus activates mTOR signaling in response to insulin/IGF-1.

Nutrients, especially amino acids, have been proposed to activate mTORC1 either via inhibition of TSC1/2 complex [92] or via stimulation of Rheb [279]. hVPS34, a class III PI3K (converts phosphatidylinositol to phosphatidylinositol-3-phosphate) have also been reported to signal amino acid availability to mTORC1 independently of the TSC1-2/Rheb axis [37,223]. TORC1 senses the cellular energy status through AMP-activated protein kinase (AMPK). AMPK is activated in response to low cellular energy (high AMP/ATP ratio). Activated AMPK then down-regulates energetically demanding processes like protein synthesis and stimulates ATP-generating processes such as fatty acid oxidation. Activated AMPK directly phosphorylates TSC2 at sites different than Akt phosphorylation sites and enhances its GAP activity, leading to inhibition of mTORC1 signaling. The tumor suppressor LKB1 acts as the upstream activator kinase for AMPK. Interestingly, it has been suggested that Akt activates mTORC1 not only by direct phosphorylation and inactivation of TSC2 but also by maintaining high ATP levels, thereby causing decrease in AMP/ATP ratio and turning off AMPK mediated activation of TSC2 [73].

Cells regulate environmental stress, such as hypoxia and low energy, by down-regulating energy demanding processes and arresting growth. Under hypoxia, TOR signaling is inhibited thereby down-regulating protein synthesis. The signal for hypoxia is mediated to mTORC1 via REDD1 and REDD2 [33,252] whose expression is up-regulated by the transcription factor HIF1. REDD acts upstream to TSC1/2 and downstream to Akt to inhibit mTORC1. REDD also acts independent of LKB1-AMPK pathway to regulate mTORC1. Other stresses like DNA damage is linked to mTORC1 through p53 [74]. p53 activated by DNA damage down-regulates mTORC1

via the AMPK-TSC2 pathway. A reducing environment is signaled to mTORC1 through the redox sensor in the FATC domain of mTOR [65,264].

Other than Akt and AMPK, TSC2 is also regulated through phosphorylation by the Ras/MAPK/Erk pathway. Ras activates Raf/MEK1/2-Erk1/2 cascade. Activated Erk1/2 then directly phosphorylates TSC2 at sites that differ from the Akt target sites and inactivates TSC1/2 complex [199]. MAPK activates RSK1 which phosphorylates and inhibits TSC2 causing activation of mTORC1 signaling [259].

Thus, different growth and stress pathways converge at the TSC1-TSC2 complex to regulate mTORC1 signaling. The best studied mTOR targets are the translation regulators S6K1 and 4EBP1. Both S6K and 4EBP1 have a conserved five amino acid sequence called the TOR signaling (TOS) motif. The TOS motif found at the N-terminus of S6K (Phe-Asp-Ile-Asp-Leu) and in the C-terminus of 4EBP1 (Phe-Glu-Met-Asp-Ile) is necessary for phosphorylation of these proteins by mTORC1 *in vivo* [267]. By phosphorylating these two substrates, mTOR regulates translation initiation. S6K1 belongs to the AGC family of protein kinases and needs to be phosphorylated at its C-terminus hydrophobic motif as well as a site in the T loop of its kinase domain. mTORC1 phosphorylates thr389 in the hydrophobic domain of S6K1. Activated S6K1 phosphorylates and activates the 40S ribosomal protein S6 which plays a role in translation of the 5' tract oligopyrimidine (5'TOP) mRNA. These mRNAs encode for ribosomal proteins and elongation factors, thus playing a role in increasing the translation capacity in general. S6K also phosphorylates the elongation factor 2 kinase (eEF2K) [306]. However, some reports have suggested that S6K or S6 are not required for TOP mRNA translation [239,261]. mTORC1 also promotes cap-dependent translation by phosphorylating and inactivating the 4EBP1 inhibitory

protein. This results in release of eIF4E, which can then associate with eIF4G to stimulate translation initiation.

Apart from regulating translation through S6K and 4EBP1, mTORC1 also regulates ribosomal biogenesis by regulating rRNA gene transcription. mTORC1 plays a role in gene expression by regulating transcription of metabolic and biosynthetic pathway genes through URI (unconventional prefoldin RPB5 interactor). mTORC1 has been recently reported to regulate the self renewal processes, autophagy, by inhibiting the protein kinase ATG1 and also by regulating trafficking of nutrient transporters thereby promoting uptake of glucose, amino acids, lipoproteins and iron [73,209,317]. Furthermore, Kim et al. have shown that mTORC1 may regulate adipogenesis, since rapamycin prevents adipocyte differentiation and lipid accumulation [159]. Although the mechanism by which mTORC1 regulates adipogenesis is not clear, it is believed that the nuclear receptor PPAR γ may be involved. Interestingly, SIRT1 regulates adipogenesis and lipid metabolism by regulating PPAR γ and other factors.

Recent studies suggest that constitutive activation of TSC-mTOR-S6K1 signaling induces a negative feedback loop to attenuate PI3K-PDK1-Akt pathway via phosphorylating and inhibiting IRS [117,272,297]. S6K1, in particular, regulates IRS1 both at the transcriptional level as well as through phosphorylation, thereby abolishing IRS1 adaptor function [297]. It was shown that loss of TSC1 or TSC2 in mouse embryonic fibroblasts (MEFs) or *Drosophila*, leads to a strong inhibition of insulin-mediated PI3K signaling [32]. Interestingly, S6K1 has also been found to phosphorylate mTOR at Thr2446 and Ser2448. S6K mediated mTOR phosphorylation is dependent on PI3K and is increased in response to constitutively active Akt, indicating a feedback loop [50,125]. The effect of phosphorylation of mTOR at Thr2446/Ser2448 on mTORC1 signaling is yet to be identified.

Detailed analysis of the mTOR pathway has revealed that mTORC1 signaling is tightly linked to tumorigenesis. Aberrantly high mTORC1 activity appears to be responsible for many cancers and hamartoma syndromes (characterized by benign tumors). The specific mTOR inhibitor rapamycin and its various derivatives are currently under clinical trial as cancer drugs. Rapamycin has also been used as an allograft rejection drug as it interferes with T cell activation, thereby acting as an immunosuppressive agent in renal, liver and cardiac transplantation. Recently, inhibitors of mTOR are also being tested for treatment against several autoimmune disorders such as rheumatoid arthritis, psoriasis, multiple sclerosis and Parkinson's disease [321]. mTOR inhibitors are also being considered for cardiac hypertrophy, a major risk factor for heart failure, since overgrowth of cardiomyocytes is dependent on the PI3K-mTORC1 pathway [135,292].

Lifespan extension has been linked with phenotypes including stress resistance, metabolic rate, lipid level, reproductive capacity and body size [8,194,226], which are affected by CR. The molecular mechanism by which CR extends lifespan is not yet clear, however, evidence suggest that IGF-1/TOR pathways may play a key role. First, CR reduces the plasma IGF-1 levels [31]. Second, the CR mediated lifespan extension of *Drosophila* and yeast is not increased further by mutations in the IGF/TOR pathway [150]. Finally, the TOR pathway acts as a nutrient sensor which, like SIRT1, makes it a good candidate to mediate the effect of CR on lifespan. Both mTOR and SIRT1 have been shown to be involved in pathways eliciting these physiological attributes, suggesting that these pathways may possibly coordinate or intersect with each other. Despite a conceivable link between SIRT1 mediated regulation of metabolism, longevity and age related diseases, and mTOR mediated regulation of pathways involved in growth signaling, longevity and diseases, no direct link has been established yet between these

two important regulators. We wished to investigate a possible overlap between mTOR and SIRT1 with regards to stress resistance and cell growth. In the present section we demonstrate for the first time that SIRT1 indeed regulates mTOR signaling through TSC2 and this regulation is potentially mediated through SIRT1's deacetylase activity.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture and treatment and transfection

HeLa, 293T and Jurkat cells were purchased from ATCC and maintained in DMEM media (Dulbecco's modified Eagle's media) supplemented with 10% heat inactivated fetal bovine serum and 100U penicillin, 100U streptomycin and 0.25 μ g/ml amphotericin B (antibiotic-antimycotic; GIBCO # 15240-062), Hepes buffer and 2mM L-glutamine. Cells were cultured at 37⁰C, 19% O₂ and 5% CO₂. The SIRT1-wild-type and SIRT1 null mouse embryonic fibroblasts were a gift from Dr. Michael McBurney, University of Ottawa, Canada. These cells were also maintained in the DMEM growth media. SIRT1 MEFs were cultured at 37⁰C, 15% O₂ and 5% CO₂. For various treatments, cells were seeded at about 80% confluency in culture dishes and cultured for overnight in normal growth media. Next day, cells were treated treatment media such as: leucine negative media (RPMI 1640, Cellgro, # 10-040-CV), glucose negative media (DMEM), serum negative media (Normal growth media without FBS), 2 μ g/ml tunicamycin, TM (SIGMA # T7765) in normal growth media, 500 μ M H₂O₂ in normal growth media, 200nM insulin in normal growth media, rapamycin (SIGMA# R0395), resveratrol (SIGMA # R5010) and nicotinamide. The solvent for chemicals like TM, insulin and H₂O₂ were used in similar concentration for mock-treatment where ever possible. Cells were harvested by treating cells with trypsin-EDTA (Cellgro, # 25-053-C1) for 2mins in 37⁰C incubator. Cells were washed by re-suspending cells in growth media and centrifugation at 5000g in a table-top centrifuge.

For transfection, cells were seeded at ~80% confluency in DMEM growth media without antibiotic-antimycotic, and cultured overnight. Cells were transfected the next day using Lipofectamine2000 transfection reagent (Invitrogen # P/N 52887) according to manufacturer's

protocol. The transfection media on cells were replaced with normal growth media 6hrs post-transfection. The next day cells were treated as required for specific experiments and harvested for analysis.

4.3.2 Immunofluorescence Microscopy

HeLa cells were seeded on sterilized (in 99% ethanol) coverslips at 60 % confluency in a 6 well plate and cultured in normal growth media for overnight. Cells were fixed in 2% paraformaldehyde for 15 min at room temperature. Immunostaining was performed by incubating fixed cells in 1:1000 diluted anti-SIRT1 (mouse, clone 2G1/F7, Upstate) antibody for 1hr, followed 45 min incubation with secondary antibody conjugated with alexa-fluor488 (anti-mouse) at RT. DAPI (blue) was used for nuclear staining. Following the secondary antibody treatment, cells were washed with PBS and the coverslips were mounted on slides in Vectashield (Vector Labs, Burlingame, CA) for fluorescent microscopy. Samples were examined by using an Eclipse E600 microscope (Nikon). Pictures were taken at 40X magnification.

4.3.3 F-actin staining using rhodamine-phalloidin

Cells were seeded in a 6 well plate on sterilized cover-slips and grown for 24 hr. Cells were then fixed in 2% formaldehyde for 15 mins at room temperature. Rhodamine-conjugated phalloidin (Molecular probes) was used for F-actin staining and DAPI was used as nuclear stain. The cells were examined for fluorescence under fluorescent microscope and pictures were taken at 40X magnification.

4.3.4 Whole cell lysate preparation and protein quantification

Whole cell lysates were made by lysing cells in NP-40 lysis buffer (20mM TrisHCL, pH8.0, 137mM NaCl, 10% glycerol, 1% nonidet P-40, 2mM EDTA). 1X protease inhibitor cocktail (SIGMA # P2714) was added to lysis buffer just before lysis. Cells were lysed for 20 min on ice and lysate was separated from cell debris by centrifugation for 20 min at 13000g in a micro-centrifuge at 4°C. For total protein extracts from kidney tissue, kidney was homogenized in the RIPA lysis buffer (50 mM Tris·HCl/1% Nonidet P-40/150 mM NaCl/1 mM EDTA/1 mM NaF/1 mM Na₃VO₄) and protease inhibitors (Roche Molecular Biochemicals # 1836170) and centrifuged for 20 min at 15,000 × g at 4°C. Nuclear and cytoplasmic sub-cellular fractionation was done using NE-PER fractionation kit (Pierce, #78833). Protein concentration of the cell lysates were estimated by Bradford assay. 2µl of lysate was added to 1ml BioRad protein assay reagent (#500-0006, BioRad Labs, Hercules, CA). 0, 1, 2, 3, 4, 6 and 8 µg BSA (SIGMA # A2153) from 1mg/ml stock was used to plot the standard curve. Readings were taken at 595nm wavelength.

4.3.5 Co-immunoprecipitation for detection of protein-protein association

HeLa cells were cultured in 100mm dish in normal growth media as described above. Cells were either used for endogenous or epitope-tagged overexpressed protein-protein interaction. For exogenous protein co-immunoprecipitation, cells were co-transfected with 10µg pcDNA6-flag-SIRT1 and pcDNA6-flag-HA-TSC2 expression plasmids in antibiotic free media using lipofectamine2000 transfection reagent. 24 hrs post transfection, cells were either mock treated for treated with –leucine media, 500µM H₂O₂, 10mM nicotinamide (NAM) and 100µM

resveratrol (RES) for 1hr. Cells were scraped and lysed using NP40 lysis buffer +PI. 500µg of whole cell lysate was incubated overnight with 10 µl anti-TSC2 and 2µg anti-SIRT1 (for endogenous protein CoIP) or 2µg anti- SIRT1 and 2µg anti-flag (for over-expressed protein CoIP) at 4⁰C. Anti-IgG was used as negative control for immunoprecipitation. Immunoprecipitated proteins were then pulled down by incubating lysates with 35µl ProteinA-sepharose (SIGMA # P9424) and 25µl ProteinG-agarose (SIGMA # P4691) 50% slurry at 4⁰C on a rotator for 2 hrs. The resin was pelleted by spinning the samples at 2000g for 5 mins in microcentrifuge. The precipitated resin was then washed three times in cold lysis buffer. Centrifugation during washes was carried at 2000g. After the final wash, the supernatant was carefully discarded by suction using a fine tip pipette. The beads were then resuspended in 25µl of 2X-Lamlli sample loading buffer with (BioRad # 161-0737) and boiled for 5 mins to elute the proteins. Protein sample was spun at 13000 rpm for 1 min at RT to precipitate the beads and the supernatants were loaded in 8% separating gel for SDS-PAGE. Blots were then probed for anti-SIRT1 and anti-TSC2 or anti-HA antibody. Antibodies used for immunoprecipitation are as follows: anti-SIRT1 (Upstate # 05-707), and anti-TSC2 (cell signaling technology # 3612), anti-flag-tag (Chemicon # MAB3118), anti-HA-tag (Cell Signaling, # 2367), rabbit normal IgG (SantaCruz Biotech # sc2027) and mouse normal IgG (sc-2025).

4.3.6 Western blot analysis for mTOR signaling

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the experiment, immunoprecipitated samples or 50µg lysate protein was loaded in 15% or 8%

polyacrylamide gels. Separating gels were made using Protogel 30% w/v acrylamide: 0.8% w/v bis-acrylamide (National Diagnostic# EC-890), 1.5M Tris-HCL pH8.8, 10% ammonium persulfate (APS), 10% SDS and 0.1% TEMED (BioRad # 161-0801). 4% Stacking gel was made using same above reagents, except 1.5M Tris-HCL pH6.8 instead of pH 8.8. Proteins of lower molecular weight ranging between 15-75 kD were resolved in 15% separating gels, whereas protein with higher molecular weight (100-300 kD) were resolved in 8 % separating gels. Gels were run at 10mA for one minigel or at 50V in 1X-SDS-running buffer. Precision plus dual color protein marker (BioRad # 161-0374) was used as molecular weight standard. Samples resolved on SDS-PAGE were transferred on PVDF membrane (Immobilon-P, Millipore, Bedford, MA) in a semidry transfer machine (Bio-Rad, Richmond, CA) at 15V for 1h at RT for 15% gels and 2hr at 4°C for 8% gels. Membranes were then incubated in blocking buffer (1X PBS, 5% w/v non-fat dry milk, 1% Tween-20) for 1hr at RT on a rocker. After blocking, membranes were washed three times with wash buffer (1X PBS, 1% Tween-20) for 5mins each. The washed membranes were then incubated with primary antibody overnight at 4°C on a rocker. Primary antibody dilutions used were as follows: 1:2000 for anti-SIRT1, anti-flag-tag and 1:1000 for all other antibodies. Following primary antibody staining, membranes were given three washes with wash buffer (PBST), 10 mins each and incubated with corresponding HRP-conjugated-secondary antibody (1:2000) for 1 hr at RT on a rocker. Membranes were then washed three times in PBST and proteins were visualized by enhanced chemiluminescence (# NEL103, PerkinElmer Life Science, Inc). For re-probing the same blot, membranes were incubated in Restore™ western stripping buffer (Pierce # 21059) for 10-15mins, followed by three washes, 10 mins each in PBST. Blots were then blocked as mentioned above and probed with appropriate primary and corresponding secondary antibody, and visualized as described above. Antibodies used for

western blotting are as follows: anti-SIRT1 (Upstate # 05-707, 1:2000), anti-flag-tag (Chemicon # MAB3118, 1:2000), anti-PARP1 antibody (# sc-25780, 1:5000) and anti-HSP90 (sc-13119, 1:5000). All other antibodies were purchased from Cell signaling technologies; phospho-mTOR (# 2971), mTOR (# 2972), phospho-S6 (# 2211), S6 (#2217), phospho-S6K (# 9205), S6K (#9202), phospho-4EBP1 (# 9451), 4EBP1 (# 9452), phospho-4E (#9741), TSC2 (# 3612), TSC1 (#4963), Raptor (# 4978), Rheb (# 4935).

4.4 RESULTS

4.4.1 SIRT1 regulates mTOR signaling in human and mouse cells

mTOR regulates cellular and organism growth by sensing nutrient and hormonal signals. Enhanced mTOR activity is associated with an increase in cell size [268]. We observed that SIRT1 null mouse embryonic fibroblasts (MEFs) show larger cell morphology compared to wild type MEFs (Figure 4.4). This is consistent with a recently published article that documented enlarged and flattened morphology of SIRT1-inhibited or SIRT1 depleted (by siRNA) human endothelial cells [231].

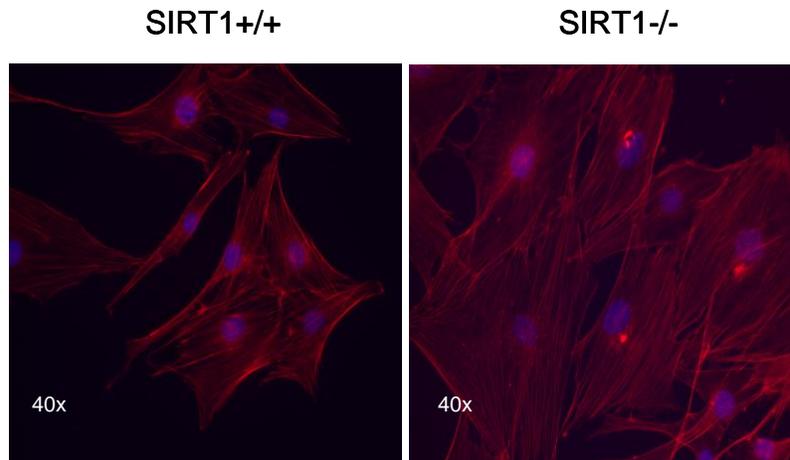


Figure 4.4 SIRT1 null mouse embryonic fibroblasts are larger in size

SIRT1 null and wild type mouse embryonic fibroblasts (MEFs) were stained for F-actin using rhodamine labeled phalloidin (red) stain. The nucleus was stained with DAPI (blue). Actin staining was visualized by fluorescent microscopy at 40X magnification.

Given that up-regulation of mTOR leads to larger cell mass and size, we investigated the activity of the mTOR pathway in WT and SIRT1 null MEFs by analysis of phosphorylation of the mTOR substrates, S6K and 4EBP1, as well as S6. Since the mTOR pathway is regulated by

nutrients and growth conditions, we examined mTOR signaling in these cells with or without stress conditions by treating the cells with no treatment or leucine starvation. As shown by higher levels of phosphorylated mTOR substrates; S6K, S6 and 4EBP1, mTOR activity remained significantly up-regulated in SIRT1 null MEFs regardless of stress condition (Figure 4.5).

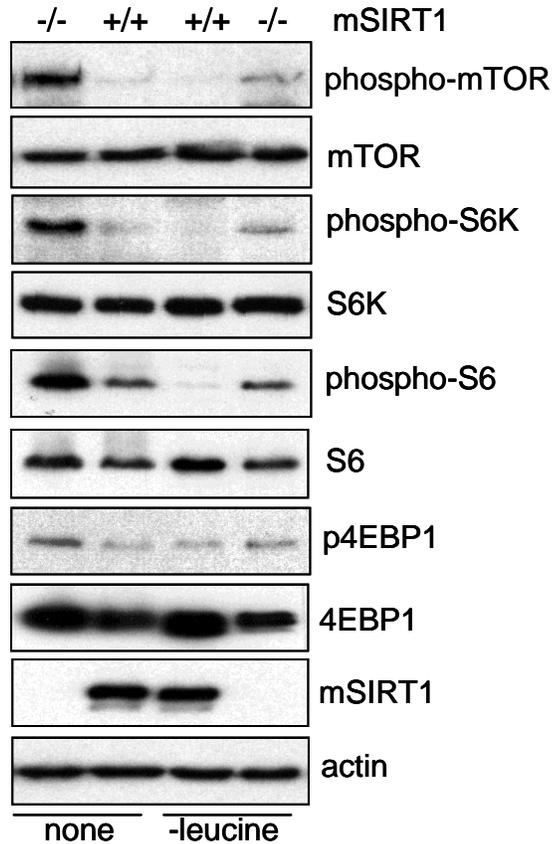


Figure 4.5 mTOR signaling is more active in SIRT1 null mouse embryonic fibroblasts

Wild-type and SIRT1 null MEFs were either mock-treated or leucine starved for 1 hr. Whole cell extracts were made using NP-40 lysis buffer followed by Western blot analysis of phosphorylation of the mTOR substrates using phospho-protein-antibody (Cell Signaling Technology) for various mTOR substrate proteins.

SIRT1 is expressed at very high levels in kidney [64]. To determine if SIRT1 regulates mTOR signaling *in vivo* in mice, kidney extracts from SIRT1 homozygous or heterozygous null mice (Figure 4.6) were examined. Consistent with the results in MEFs, extracts of kidney from

SIRT1 homozygous null and SIRT1 heterozygous null mice showed higher mTOR activity when compared with wild type mice.

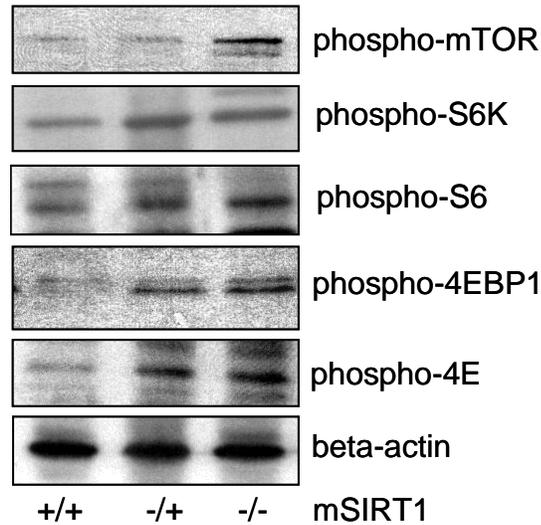


Figure 4.6 Mouse kidney from SIRT1 heterozygous or homozygous null mice show higher mTOR activity

Kidney extracts from wild-type (+/+), SIRT1 heterozygous null (+/-) and homozygous null (-/-) mice were made using Triton-X100 lysis buffer. mTOR activity was measured by western blot analysis of the phosphorylated mTOR pathway proteins.

To confirm these results in human cells, HeLa cells stably depleted in SIRT1 using shRNAi were generated by stable retroviral infection (as described in chapter 2 of the thesis). The cells were either mock-treated or treated with various different stress conditions such as amino acid stress (leucine starvation), energy stress (glucose starvation), ER stress (tunicamycin), growth factor stress (serum deprivation) and free radical stress (hydrogen peroxide stress). As shown in Figure 4.7, under these different stress conditions, mTOR activity remained significantly up-regulated in cells that had reduced levels of SIRT1 as indicated by higher phosphorylation of the mTOR substrates S6K and S6.

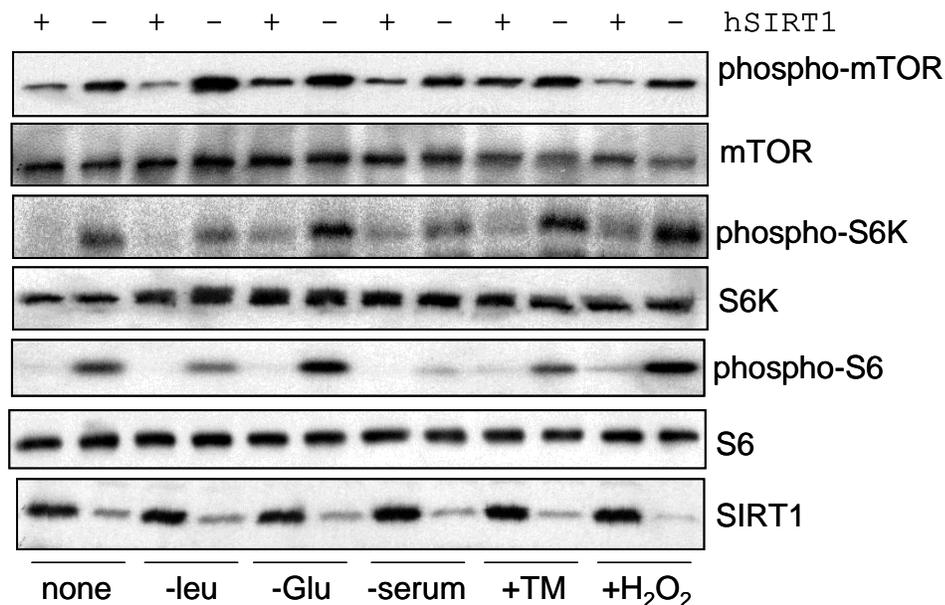


Figure 4.7 SIRT1 depleted HeLa cells show higher mTOR activity

A stable HeLa cell line depleted for SIRT1 using shRNA and corresponding control HeLa cells were either mock-treated or treated for 1 hr with indicated stress conditions [-leu: leucine deprived, -Glu: glucose deprived, -serum: serum deprived, +TM: 2ug/ml tunicamycin, +H₂O₂: 500μM hydrogen peroxide]. mTOR activity was measured by western blot analysis of phosphorylation of mTOR substrates using phospho-specific antibodies. hSIRT1: human SIRT1.

4.4.2 SIRT1 does not alter the protein expression of mTOR-complex 1 regulatory proteins.

Since SIRT1 is known to regulate gene expression of many proteins by acting as a regulator of various transcription factors, we investigated whether SIRT1 regulates mTOR signaling by regulating the expression levels of the various proteins that are critical for mTOR signaling. Upstream to mTOR complex-1 is the TSC1/TSC2 inhibitory complex which inhibits the

mTORC1 activator protein Rheb. The association of mTOR with Raptor to form the mTOR complex is critical to mTOR signaling. Using HeLa cells that were depleted for SIRT1 using shRNAi by stable retroviral infection, we examined the expression of the mTORC1 regulatory proteins. SIRT1 depleted HeLa cells and matched controls were either mock-treated or treated for one hour with various stress condition such as amino acid stress (leucine starvation), energy stress (glucose starvation), ER stress (tunicamycin), growth factor stress (serum deprivation) and free radical stress (hydrogen peroxide stress). Protein levels of TSC1, TSC2, Raptor and Rheb were then analyzed by western blot analysis. As shown in Figure 4.8, the levels of these proteins were not altered regardless of the presence of SIRT1, indicating that SIRT1 does not regulate the expression of these proteins.

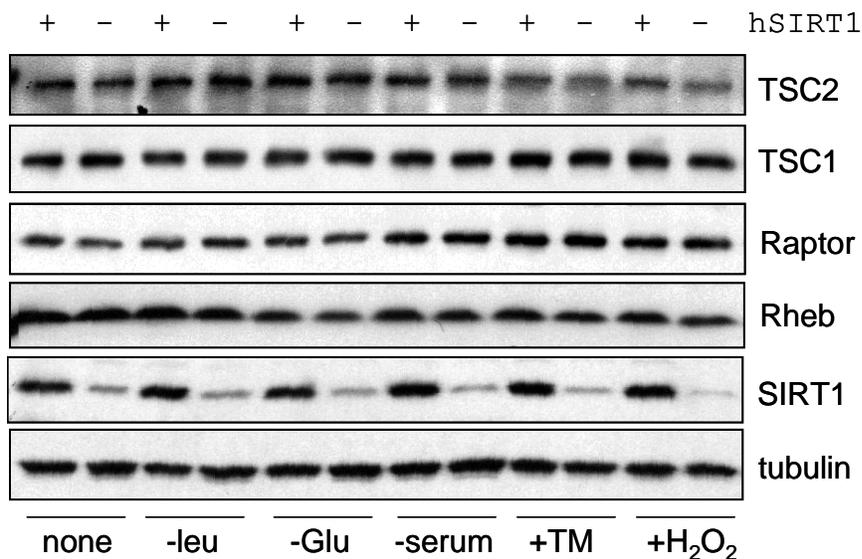


Figure 4.8 Expression level of mTOR regulatory proteins are not altered in response to presence or absence of SIRT1

SIRT1 depleted and control HeLa cells that were either mock-treated or treated for 1 hour with various stress conditions as indicated [-leu: leucine deprived, -Glu: glucose deprived, -serum: serum deprived, +TM: 2ug/ml tunicamycin, +H₂O₂: 500μM hydrogen peroxide]. Expression level of proteins up-stream activator (Rheb) and

inhibitor (TSC1/2) proteins as well as the mTOR-complex 1 (mTORC-1) Raptor, were analyzed by western blot analysis of whole cell extracts using total protein antibody.

4.4.3 SIRT1 regulates mTOR signaling up-stream of the mTOR complex-1 (mTORC1).

Rapamycin, a macrolide that is produced by the bacterium *Streptomyces hygroscopicus* acts as a specific inhibitor of mTOR complex 1 (mTORC1), but does not inhibit the mTOR complex 2 (mTORC2). Upon entering cells, rapamycin binds to its intracellular receptor, FKBP12, a cytosolic binding protein collectively called immunophilin. This complex then forms a ternary complex with mTOR and inhibits mTOR and mTOR-mediated signaling network [118].

To investigate if SIRT1 regulates mTOR signaling upstream to mTORC1, we treated control and SIRT1 depleted HeLa cells with 25nM rapamycin and examined the mTOR activity by analyzing phosphorylation of mTOR substrates. As shown in Figure 4.11, rapamycin treatment completely abolished the phosphorylation of S6K, S6 and 4EBP1 in SIRT1 depleted as well as control cells indicating that SIRT1 regulates mTOR upstream of mTORC1.

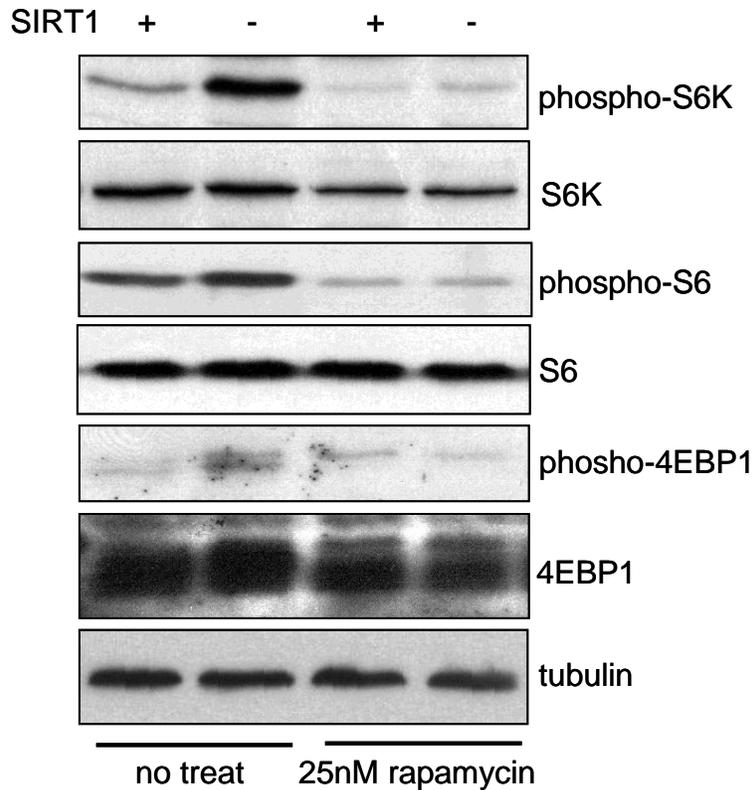


Figure 4.9 Rapamycin abrogates mTOR signaling regardless of presence of SIRT1

HeLa cells depleted for SIRT1 (-) and control matched cells (+) were treated with 25nM rapamycin for 1hr. Whole cell lysates were analyzed by western blotting for phosphorylation of various mTOR substrates. Anti-tubulin was used to show equal load for all lanes.

Upstream to mTORC-1 is the inhibitory complex TSC1/TSC2. mTOR is downregulated by TSC1/TSC2 complex through its regulation of the small GTP-binding protein Rheb. TSC2 acts as a GTPase activating protein for Rheb, an upstream activator of mTOR, thereby inactivating Rheb and inhibiting mTOR signaling [136]. It has been shown that TSC2 null cells show persistently upregulated mTOR signaling whereas over-expression of TSC1 and TSC2 leads to mTOR inhibition [244,324]. Since, we observed that SIRT1 acts as an inhibitor of mTOR signaling, we wished to investigate, if SIRT1 null MEFs respond similarly as TSC2 null MEFs in response to rapamycin treatment. Wild-type and TSC2 null or SIRT1 null MEFs were

treated with 25nM rapamycin and mTOR signaling was examined by phosphorylation of various mTOR substrates. Consistent with published reports on TSC2 null MEFs and our data in SIRT1 depleted HeLa cells, treatment of both TSC2^{-/-} and SIRT1^{-/-} MEFs with rapamycin resulted in complete abrogation of the elevated mTOR activity, demonstrating that SIRT1 null MEFs are similarly sensitive to rapamycin as the TSC2 null MEFs (Figure 4.10).

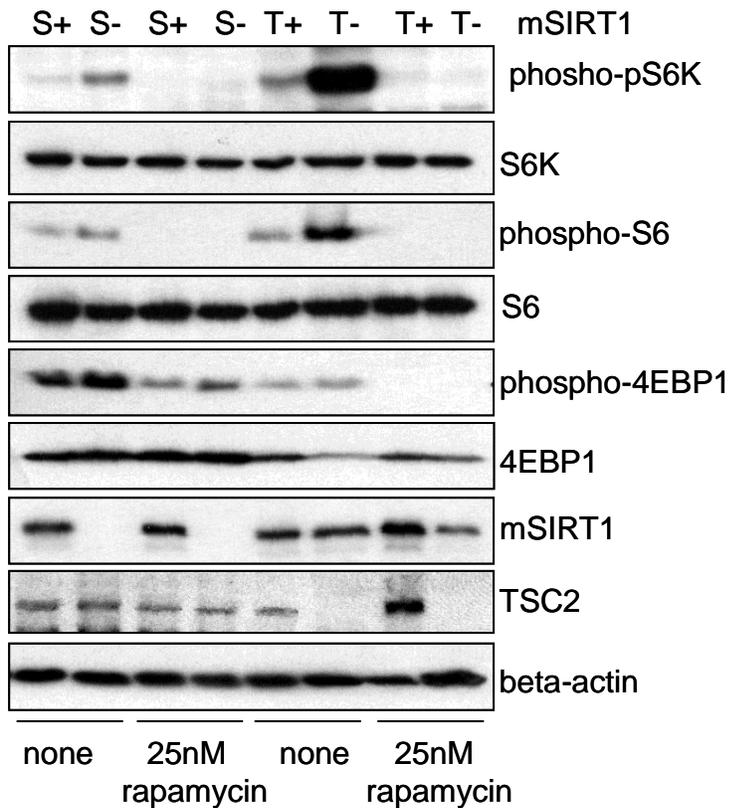


Figure 4.10 SIRT1 null mouse embryonic fibroblasts show abrogation of mTOR signaling in response to rapamycin treatment similar to the TSC2 null mouse embryonic fibroblasts

Wild-type (T+: TSC2^{+/+} and S+: SIRT1^{+/+}) and TSC2 null (T-) or SIRT1 null (S-) mouse embryonic fibroblasts were treated with 25nM rapamycin for 1hr. Whole cell lysates were analyzed by western blotting for phosphorylation of various mTOR substrates. B-actin was used to show equal loading for all lanes.

4.4.4 The catalytic activity of SIRT1 plays a role in regulating mTOR signaling

SIRT1 is a NAD⁺ dependent deacetylase whose catalytic activity plays a role in most of the known biological functions of SIRT1. Resveratrol (RES), a natural polyphenol, acts as an activator of SIRT1 that has been shown to increase lifespan in *S. cerevisiae*, *C. elegans* and *D. melanogaster* in a SIRT1-dependent manner [127,313]. Nicotinamide (NAM), a byproduct of SIRT1 catalytic reaction, non-competitively inhibits SIRT1. SIRT1 enzymes are multifunctional in that the deacetylase reaction involves the cleavage of the nicotinamide-ribosyl, cleavage of an amide bond, and transfer of the acetyl group ultimately to the 2'-ribose hydroxyl of ADP-ribose. Nicotinamide inhibits SIRT1 by intercepting an ADP-ribosyl-enzyme-acetyl peptide intermediate with regeneration of NAD⁺ (transglycosidation) [139].

To examine if the catalytic activity of SIRT1 is important for regulation of mTOR signaling, HeLa cells were treated with the SIRT1 activator resveratrol (RES) and inhibitor nicotinamide (NAM), under stress (-leucine) or growth (Insulin) conditions. mTOR signaling was measured by examining phosphorylation levels of S6 and 4EBP1. As shown in Figure 4.13, resveratrol suppressed mTOR signaling regardless of stress or growth conditions, suggesting that the catalytic activity of SIRT1 may play a role in mTOR regulation. We also examined the effect of RES and NAM on 293T cells. As shown in figure 4.11, RES and NAM had similar effects on mTOR signaling in 293T cells as they had in HeLa cells. Interestingly however, we observed higher mTOR signaling even under leucine starvation in 293T cells, which was completely abolished by RES treatment.

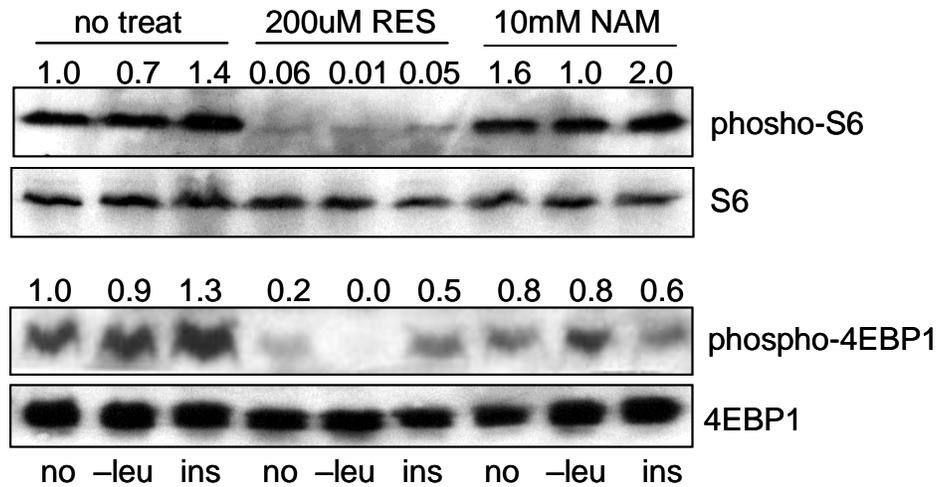


Figure 4.11 SIRT1 activator suppresses and inhibitor activates mTOR signaling in HeLa cells

HeLa cells were either mock-treated or treated for 1 hr with -leucine media, 200nM insulin and 100µM resveratrol (RES) or 10 mM nicotinamide (NAM) as indicated. mTOR signaling was analyzed by western blotting for phosphorylation of the mTOR substrate proteins.

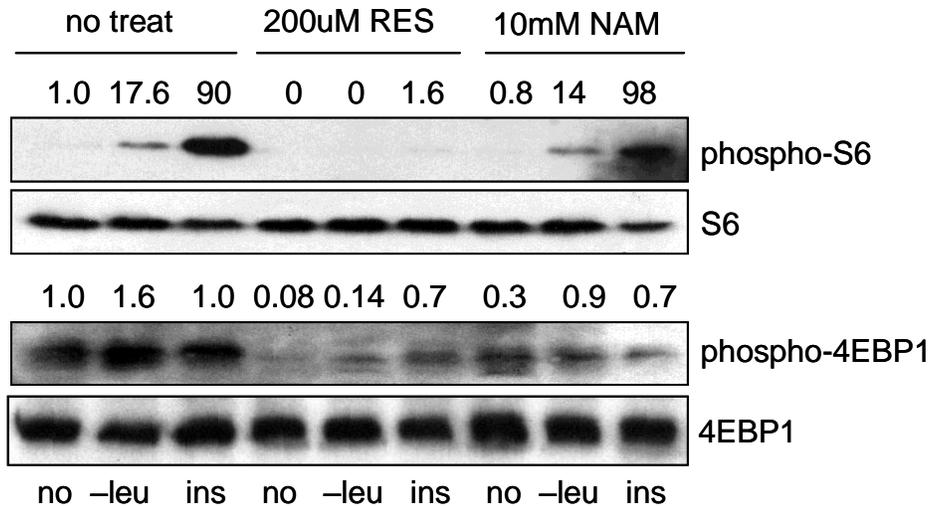


Figure 4.12 SIRT1 activator suppresses mTOR signaling in 293T cells

293T cells were either mock-treated or treated for 1 hr with -leucine media, 200nM insulin and 100µM resveratrol (RES) or 10 mM nicotinamide (NAM) as indicated. mTOR signaling was analyzed by western blotting for phosphorylation of the mTOR substrate proteins.

To further confirm that the catalytic activity of SIRT1 is important for regulation of mTOR signaling, control and SIRT1-depleted HeLa cells were treated with the SIRT1 inhibitor nicotinamide (NAM) and mTOR signaling activity was examined. Consistent with the previous observation, we observed upregulation S6 and 4EBP1 phosphorylation in the NAM treated control cells. This further demonstrates that inhibition of SIRT1's catalytic activity upregulated mTOR signaling in control cells making them behave similar to the SIRT1 depleted cells (Figure 4.13).

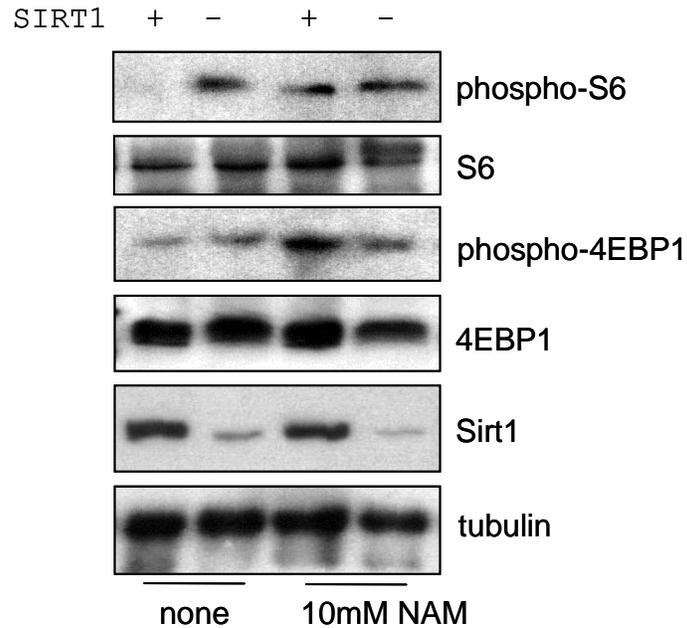


Figure 4.13 SIRT1 inhibitor activates mTOR signaling in control but not in SIRT1 depleted HeLa cells

SIRT1-depleted (-) and control (+) HeLa cells were either mock-treated or treated with 10mM nicotinamide for 1 hr. mTOR signaling was analyzed by western blotting for phosphorylation of the mTOR substrate proteins. Anti-tubulin antibody was used to show equal load loading in all lanes.

4.4.5 SIRT1 inhibits mTOR signaling by acting through the TSC2

TSC1/2 antagonizes the mTOR signaling pathway via stimulation of GTP hydrolysis of the mTORC1 upstream activator protein, Rheb [187]. TSC1/2 thus plays a pivotal role in mediating growth factors, nutrient and energy sensing signals to mTOR-dependent targets. Since our results suggested that SIRT1's inhibitory effect on mTOR signaling is upstream of mTORC1, we investigated if SIRT1 mediated down-regulation of mTOR signaling was TSC2 dependent. As shown in figure 4.14, while SIRT1 inhibitor NAM could stimulate, and the SIRT1 activator RES could inhibit S6 phosphorylation in wild type MEFs, they were ineffective in TSC2 null MEFs suggesting that SIRT1 potentially mediates its effects through TSC2.

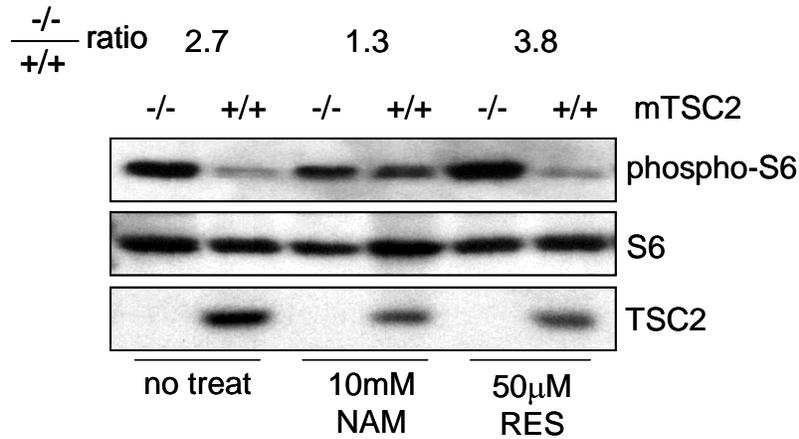


Figure 4.14 SIRT1 activator can not suppress mTOR signaling in TSC2 null MEFs

Wild-type and TSC2 null MEFs were either mock-treated or treated with 10 mM nicotinamide or 50µM resveratrol for 1 hr. mTOR signaling was analyzed by western blotting for phosphorylation of the mTOR substrate protein S6.

Resveratrol has been reported to have SIRT1 independent effects. For example, resveratrol inhibits the insulin induced Akt and MAPK activation in a SIRT1 independent

manner [325]. Resveratrol also activates AMPK, an activator of TSC1/2 complex, in Neuro2a cells and primary neurons in a LKB1 dependent but SIRT1 independent way [66]. Since, resveratrol is documented to regulate certain pathways independent of SIRT1, in order to confirm that the effect of resveratrol on mTOR pathway, shown in figure 4.15 was SIRT1 dependent, we examined the effect of resveratrol in wild-type versus SIRT1 null MEFs. Our results show that resveratrol inhibits S6 phosphorylation in wild-type, but not in SIRT1 null MEFs, demonstrating that the effect of resveratrol on mTOR signaling is through SIRT1 (figure 4.16).

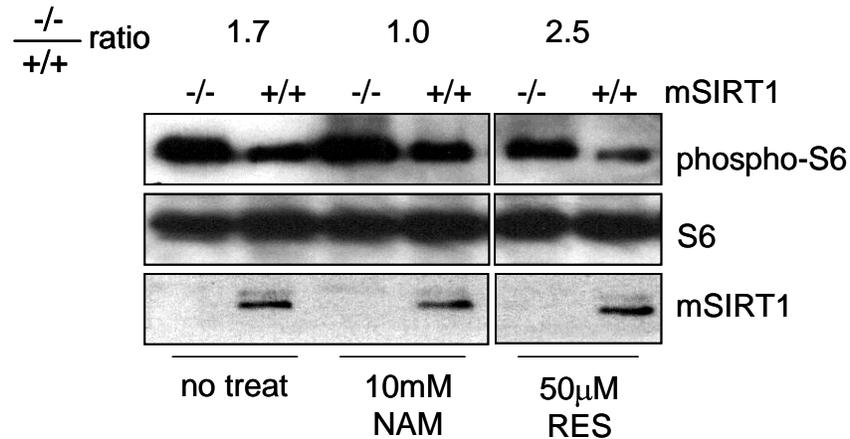


Figure 4.15 Resveratrol suppresses mTOR signaling in wild-type but not in SIRT1 null MEFs

Wild-type and SIRT1 null MEFs were either mock-treated or treated with 10 mM nicotinamide or 50µM resveratrol for 1 hr. mTOR signaling was analyzed by western blotting for phosphorylation of the mTOR substrate S6.

Furthermore, in a dose response effect of resveratrol on wild-type versus TSC2 null or SIRT1 null MEFs, we observed that while S6 phosphorylation remained high in TSC2 null MEFs regardless of increase in resveratrol concentration, the SIRT1 null MEFs showed mild reduction in S6 phosphorylation in response to higher (100µM) dose of resveratrol, suggesting a

parallel SIRT1 independent pathway through which resveratrol down-regulates the mTOR pathway.

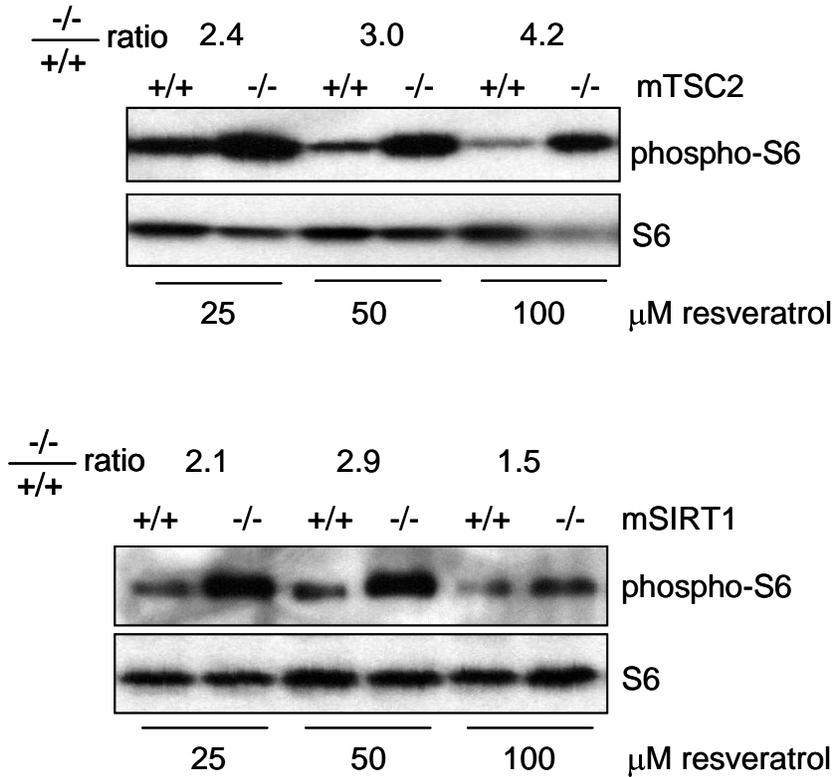


Figure 4.16 Resveratrol suppresses mTOR signaling at higher dose in SIRT1 null MEFs but not in TSC2 null MEFs

Wild-type, TSC2 null and SIRT1 null MEFs were either mock-treated or treated with 25, 50 or 100μM resveratrol for 1 hr. mTOR signaling was analyzed by western blotting for phosphorylation of the mTOR substrate S6.

4.4.6 SIRT1 associates with TSC2 in HeLa cells

Given that the effects of resveratrol and nicotinamide on mTOR regulation were both SIRT1 and TSC2 dependent, we investigated by co-immunoprecipitation if these two proteins associated

with each other. As shown in Figure 4.17 immunoprecipitation of endogenous TSC2 resulted in co-immunoprecipitation of endogenous SIRT1 (A) and conversely, TSC2 was co-immunoprecipitated with SIRT1 (B).

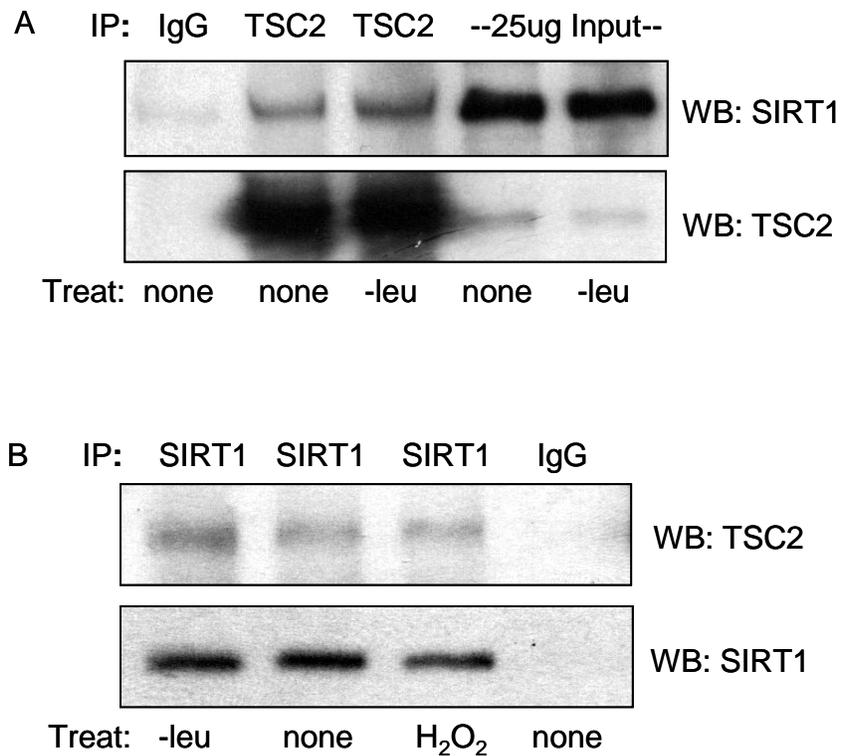


Figure 4.17 Endogenous SIRT1 and TSC2 Co-immunoprecipitate in HeLa cells

A. HeLa cells were either mock-treated or leucine deprived for 1hr. Whole cell extracts were used to immunoprecipitate endogenous TSC2. Endogenous SIRT1 was probed using anti-SIRT1 antibody by western blot analysis of the immunoprecipitated extracts. (B) Endogenous SIRT1 was immunoprecipitated from whole cell extracts of cells either mock-treated or treated for 1 hr with 0.5 mM H₂O₂ or leucine deprived media. Endogenous TSC2 was probed using anti-TSC2 antibody by western blot analysis of the immunoprecipitated extracts.

To further confirm the association of SIRT1 and TSC2, tagged versions of SIRT1 and TSC2 proteins were over-expressed in HeLa cells. Cells were then treated with no treat, growth (200nM insulin) or stress (-leucine) conditions or with SIRT1 activator, resveratrol (100µM) or inhibitor, nicotinamide (10mM). As shown in Figure 4.18, epitope tagged SIRT1 co-immunoprecipitated with TSC2 (A) and vice-versa (B), verifying the association of TSC2 and SIRT1 regardless of stress or growth condition and SIRT1's catalytic activity.

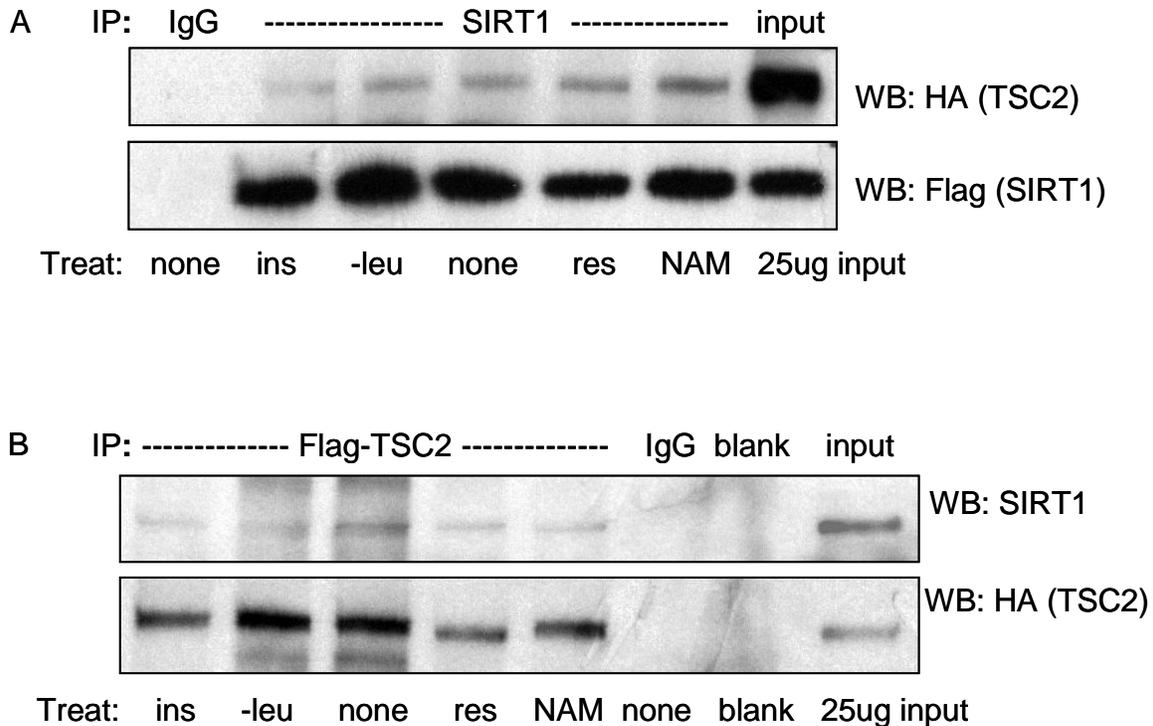


Figure 4.18 Exogenously expressed SIRT1 and TSC2 co-immunoprecipitate in HeLa cells

HeLa cells were co-transfected with pcDNA6-flag-SIRT1 and pcDNA6-HA-flag-TSC2 expression plasmids. 24 hrs post transfection, the cells were either mock-treated or treated with 200nM insulin, -leucine media, 100µM resveratrol or 10mM NAM for 1 hr. SIRT1 and TSC2 were co-immunoprecipitated by the epitope tag antibody as indicated and the immunoprecipitated extracts were analysed for SIRT1 and TSC2 by western blot analysis.

Since SIRT1 and TSC2 associated with each other, we attempted to examine if TSC2 is an acetylated protein by immunoprecipitating TSC2 and probing for acetyl-lysine (data not shown). Our results did not show any significant acetylation of TSC2. However, we can not rule out the possibility that TSC2 or any other protein in the complex was acetylated in response to stress or growth conditions. Also, interestingly, our co-immunoprecipitation data showed slightly enhanced association of mTOR and SIRT1 under stress condition (-leu) when compared to growth condition (Insulin).

4.5 DISCUSSION

SIRT1 is known to play a role in lifespan extension, age related diseases and cellular stress response by regulating various targets involved in aging related pathways such as caloric restriction and insulin/IGF-1 signaling. mTOR signaling which is a downstream component of the insulin/IGF-1 signaling as well as some other growth, nutrient and stress related signaling pathways, has been implicated in a wide spectrum of human diseases and in longevity in lower organisms.

Rapamycin and related inhibitors of the TOR signaling pathway, are being considered as a potential drug against a number of physiological conditions that are more prevalent in older age, such as cancer, type 2 diabetes, cardiovascular and neurological disorders. Soon after its discovery in the early 1970s, rapamycin was shown to be a potent anti-cancer drug. Numerous human cancers have been shown to contain mutations in genes that encode components of the P13K-mTOR signaling network [106]. By inhibiting mTOR signaling, rapamycin suppresses cell proliferation, tumor angiogenesis and cellular resistance to the hypoxic tumor micro-environment [105,128,296]. In insulin-resistant diabetes, rapamycin inhibits the sustained activation of mTOR signaling, a crucial event in IRS irresponsiveness to insulin, thereby restoring IRS sensitivity to insulin [272]. Rapamycin also inhibits adipocyte differentiation and fat accumulation by inhibiting mTOR mediated expression and activity of PPAR- γ [51,159], thereby contributing to leanness of animals, which is one of the physiological changes elicited in diet restricted longer living animals. Cardiac hypertrophy, the leading cause of heart failure, is characterized at a cellular level by increase in cell size and protein synthesis. It has been shown that inhibition of mTOR by rapamycin prevents cardiac hypertrophy in mouse models. Moreover, stress-induced cardiac hypertrophy has been shown to be mTOR dependent [85,208].

Various mTOR inhibitors are being considered for a number of human neurological disorders such as Huntington's, Alzheimer's and Parkinson's diseases. It has been shown that rapamycin induces autophagic responses to clear up neurotoxic peptides and attenuate neurodegeneration in these diseases [19,186,250].

Interestingly, SIRT1 and its activator resveratrol have also been implicated in the diseases of aging: cancer, metabolic syndromes and neurological disorders. SIRT1 has been shown to have both oncogenic as well as tumor suppressor properties. On one hand, by acting as an anti-apoptotic factor through p53, Ku70, Foxo, HIC and E2F, SIRT1 prevents cell death and promotes tumorigenesis [34,48,55,198,216,302,305], while on the other hand it sensitizes breast and prostate cancer cells to TNF α -induced apoptosis by inhibiting NF- κ B [91]. It has also been shown to deacetylate the androgen receptor, thereby suppressing its potentially oncogenic signaling and protecting prostate cancer cells from growing in response to dihydrotestosterone [88]. Metabolic syndromes (diabetes, hypertension and cardiovascular diseases) are often age related disorders which result from dysregulated metabolism leading to aberrant glucose and lipid homeostasis. By promoting fat mobilization [241], insulin secretion [27,217,255] and inhibiting glycolysis [255], SIRT1 may potentially play a role in treating these metabolic syndromes. A growing list of evidence suggests SIRT1's neuro-protective functions. SIRT1 has been shown to be involved in NAD⁺ dependent axonal protection [7]. The NAD⁺ biosynthetic enzyme (NMNAT1), which activates SIRT1 is responsible for delayed axonal degeneration in Wallerian degeneration slow mice. SIRT1 has been shown to reduce neurotoxicity in Alzheimer's disease by activating α -secretase activity and protecting against A β -neurotoxicity by inhibiting NF- κ B signaling [46,248]. Furthermore, the SIRT1 activator, resveratrol reduces

neuronal cell death in Huntington disease model, whereas SIRT1 inhibitor sirtinol blocks it [236].

Thus, SIRT1 and mTOR signaling show an uncanny relationship not only with regards to promoting longevity, but also with regards to their roles in many age related physiological responses and diseases. It appears that these two proteins act in opposite ways to regulate aging and related diseases. While it still remains to be elucidated as to how these two proteins regulate longevity in mammals, the inverse relationship of these proteins with regards to each others' roles in regulating the aging processes indicate a potential link between the two. In our study, we demonstrate for the first time that these two proteins, involved intricately in cellular processes related to stress resistance, cell survival and longevity, are indeed interconnected.

Coordinated increases in both cell number and cell size contribute to the growth of an organ or whole organism [58]. Fingar et al. showed that cell growth and cell cycle progression are separable and distinct processes in mammalian cells and that growth to appropriate cell size requires mTOR- and PI3K-dependent signals [77]. Whereas in yeast the control of cell size seems primarily to reflect nutrient conditions, in multicellular organisms both nutrients and growth factor signals coordinate cell, organ, and organismal growth. Regulation of cell size by mTOR through translation regulation and ribosome biogenesis via S6K1 and 4EBP1 is an evolutionarily conserved function of TOR. We observed that the SIRT1 knock-out mouse embryonic fibroblasts (MEFs) showed larger cell size and mass when compared to the wild-type MEFs. Since mTOR is known to regulate cell size in organisms ranging from yeast, flies, mice to mammalian cells, we investigated the mTOR activity in wild-type and SIRT1 null MEFs. By examining the downstream substrates of mTOR known to regulate cell size and mass, S6K1 and 4EBP1, we demonstrated that the SIRT1 null MEFs have higher mTOR signaling.

Normal adult kidney has been shown to have low mTOR activity. Hyperactivity of mTOR is considered to be the cause for polycystic kidney disease, a common cause of renal failure, which results from mutation in the large transmembrane protein polycystin-1. It is believed that the cytoplasmic tail of polycystin-1, which interacts with TSC2, in turn interacts with mTOR, keeping it in an inactive form [215,274]. To confirm if mTOR signaling is increased in response to absences of SIRT1 *in vivo*, we examined SIRT1 heterozygous or homozygous null mouse kidney tissue for mTOR activity. Consistent with the data in MEFs, we observed higher phosphorylation of the mTOR substrates in SIRT1^{+/-} and ^{-/-} kidney tissues, suggesting that SIRT1 negatively regulates mTOR signaling *in vivo*. Interestingly, SIRT1 has been shown to have protective functions in kidney by inhibiting TGF β and oxidative stress induced apoptosis of glomerular mesangial cells by acting through Smad7 and p53, respectively [170,171]. Our results indicating that SIRT1 suppresses mTOR activity could be another novel mechanism by which SIRT1 may be involved in conferring protection to renal cells against damage and disease.

We also demonstrated that SIRT1 downregulates mTOR in human cells and that the total protein levels of the important mTOR pathway proteins such as TSC1, TSC2, mTOR, Raptor and Rheb are not affected by absence of SIRT1 suggesting that SIRT1 probably does not regulate the mTOR signaling by affecting the mTOR pathway protein expression. mTOR signaling is activated by growth factors and nutrients, and inactivated by stress and starvation. We further demonstrate that even under stress conditions such as leucine starvation, when mTOR signaling is expected to be downregulated, SIRT1 depleted cells show higher mTOR activity, indicating that SIRT1 may be a crucial factor for maintaining normal mTOR signaling in response to growth or stress signals.

Rapamycin, the specific inhibitor of mTOR complex-1 is being considered as a therapeutic chemical in diseases or conditions that are caused by aberrant mTOR activity. Rapamycin is believed to inhibit the active mTOR-raptor complex, thus inhibiting mTOR kinase activity for its downstream substrates S6K1 and 4EBP1. We found that while SIRT1's absence resulted in upregulation of mTOR activity, rapamycin treatment abrogated the upregulated mTOR signaling in wild-type as well as SIRT1 depleted HeLa cells and mouse embryonic fibroblasts. This suggests that SIRT1 potentially imparts its regulation on mTOR signaling upstream of the point where rapamycin acts, that is upstream to mTOR complex-1. Interestingly, TSC2, which acts in an inhibitory TSC1/TSC2 complex upstream of mTOR complex-1, acts similar to SIRT1 in that, TSC2 null MEFs have been shown to have larger cell size and mass [244,245,258], and TSC2 null MEFs are sensitive to rapamycin as well as the wild-type MEFs suggesting that SIRT1 may potentially mediate its effect on mTOR through the TSC1/2 complex.

SIRT1 is a NAD⁺ dependent deacetylase whose catalytic activity has been shown to be responsible for most of its functions. We therefore investigated if the same was true for SIRT1 mediated regulation of mTOR signaling. Using the chemical activator and inhibitor of SIRT1 in HeLa and 293T cells, we demonstrated that the catalytic activity of SIRT1 was critical for regulating mTOR signaling. Also, the control-RNAi HeLa cells showed upregulated mTOR activity similar to SIRT1-RNAi HeLa cells when treated with the SIRT1 inhibitor nicotinamide (NAM), confirming that SIRT1 regulates mTOR activity through its deacetylase function. Since, our results suggested that SIRT1's deacetylase function is important for mTOR signaling, we also attempted to examine if TSC2 is an acetylated protein. Our analysis by western blotting TSC2 immuno-precipitated lysates for anti-lysine antibody could not detect acetylation of TSC2.

However, it cannot be ruled out that TSC2, or any other protein in the complex, is an acetylated protein and a potential substrate for SIRT1.

Since TSC2 null MEFs show high mTOR activity, similar to the SIRT1 null MEFs, we investigated if the SIRT activator (resveratrol) could suppress the upregulated mTOR activity in TSC2 null MEFs and SIRT1 inhibitor could block it. Our result showed that while the SIRT1 activator could not suppress upregulated mTOR activity in TSC2 null MEFs, the SIRT1 inhibitor could actually increase mTOR activity in the TSC2 positive wild-type MEFs. However, the upregulated mTOR activity in TSC2 null MEFs was not further enhanced by SIRT1 inhibitor, indicating that SIRT1 potentially acts through the TSC1-TSC2 complex to downregulate mTOR signaling. Furthermore, since, resveratrol has been reported to show SIRT1 independent effects on insulin-signaling by acting through the LKB-AMPK and MAPK-ERK pathways [66,325], we investigated whether the effect of resveratrol on mTOR activity in our experiments were through SIRT1 or independent of SIRT1. Using resveratrol in wild-type and SIRT1 null MEFs, we demonstrated that the suppressive effect of resveratrol on mTOR activity was, at least in part, through SIRT1. Our results show that while resveratrol could suppress mTOR activity in wild-type MEFs, it could not do so in SIRT1 null MEFs, showing SIRT1 was needed for resveratrol's action. Furthermore, in a dose response experiment, we demonstrate that at higher doses, resveratrol can suppress mTOR activity even in the absence of SIRT1, which is consistent with the fact that resveratrol feeds in to mTOR signaling through multiple pathways, SIRT1 being one of them. The same dose of resveratrol (50 μ M), however, could not suppress upregulated mTOR in TSC2 null MEFs, again consistent with the fact that the mTOR inhibitory complex is regulated by multiple parallel upstream pathways, such as via AMPK and REDD1. Our data in SIRT1 depleted HeLa cells demonstrate that SIRT1 downregulates the mTOR signaling

irrespective of stress type, indicating its crucial role in maintenance of optimum mTOR signaling in response to extracellular stimuli. The effect of high dose resveratrol has been shown under no stress condition in MEFs. It is, however, possible that SIRT1 may play a more important role in regulating mTOR under stress conditions, and hence the effect of resveratrol under stress conditions may potentially be fully dependent on SIRT1. Also, it is conceivable that the potential role of SIRT1 in regulating mTOR signaling may vary qualitatively depending on the specific stress stimuli, thereby either regulating mTOR signaling fully or in part by itself.

After confirming that SIRT1 mediates its effect on mTOR signaling through TSC2, we asked if SIRT1 and TSC2 associate with each other in mammalian cells. Results from our co-immunoprecipitation experiments using both endogenous as well as epitope tagged exogenously expressed proteins showed that SIRT1 and TSC2 associate with each other in HeLa cells. Furthermore, we observed that this association was present regardless of stress or growth condition, which may indicate that SIRT1 potentially plays a constitutive role in maintaining the correct signaling through mTOR complex-1 by sensing growth or stress stimuli. We also observed that the chemical activator or inhibitor of SIRT1 deacetylase activity also did not have an effect on the SIRT1-TSC2 association indicating that the catalytic activity of SIRT1 may be needed after its association with TSC2 and potentially to the mTORC1-inhibitory TSC1/2 complex.

Thus we propose a novel SIRT1 mediated regulation of the mTOR complex-1. We showed that SIRT1 downregulates mTOR signaling in human and mouse cells. SIRT1 binds to TSC2 and requires TSC2 for its inhibitory role on the mTOR pathway. We propose a model whereby SIRT1 is crucial for sensing stress stimuli to inhibit mTOR and potentially functions by

deacetylating members of the TSC1/2 complex to inhibit mTOR signaling in response to stress conditions.

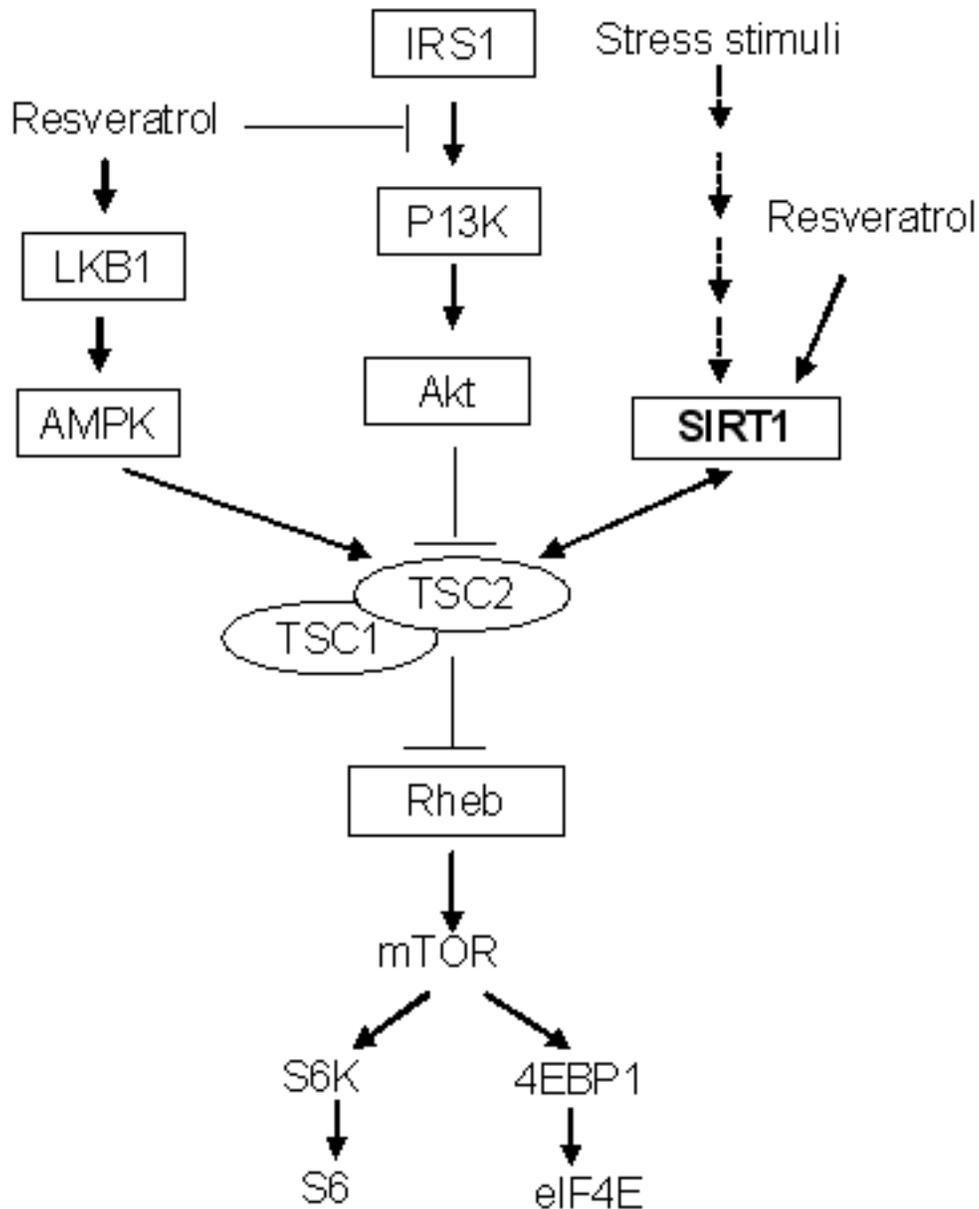


Figure 4.19 Proposed model for SIRT1 mediated regulation of mTOR

SIRT1 is important for sensing the extra-cellular stress response to down-regulate mTOR signaling when conditions are not favorable. SIRT1 associates with TSC2 and down-regulates mTOR signaling potentially by deacetylating the components of the inhibitory TSC1/2 complex.

The role of both mTOR and SIRT1 in aging diseases as well as in regulating insulin signaling suggested a potential overlap between SIRT1 and mTOR signaling mediated lifespan extension. We demonstrate for the first time that these two important pathways involved in aging and related diseases are interconnected. The novel role of SIRT1 in regulating mTOR signaling which regulates growth and proliferation could be potentially interesting from a therapeutic point of view. SIRT1 is known to be a pro-cell survival factor due to its many protective functions. Regulation of the mTOR could be another mechanism by which SIRT1 potentially plays a role against stress-induced apoptosis and aberrant cell growth under unfavorable conditions. We believe that the role of SIRT1 in regulating mTOR signaling could also be crucial in understanding SIRT1's role in mammalian aging processes. Even though SIRT1 is known to regulate the insulin signaling pathway in worms to promote longevity, it is not clear how exactly SIRT1 mediates longevity like effects in mammals. Our discovery that SIRT1 acts at a crucial regulatory point; TSC1/2 complex in the mTOR pathway, is one step towards the elucidation of SIRT1's direct role in regulating the insulin-signaling pathway in mammals in response to stress stimuli and potentially longevity.

5.0 OVERALL DISCUSSION AND CONCLUSIONS

This dissertation focuses on the protein SIRT1, which is a deacetylase enzyme that regulates a wide range of cellular processes by de-acetylating various protein substrates. These various protein substrates that SIRT1 regulates mediate a myriad of cellular processes ranging from metabolism, growth and differentiation, development, endocrine signaling and apoptosis. SIRT1 is also known as a lifespan determinant in lower organisms, potentially acting through calorie restriction. Although, SIRT1 has not been directly shown to regulate lifespan in mammals, various studies in knock-out mouse models suggest that SIRT1 may play an important role in cell survival and longevity. Further studies identifying various SIRT1 associating proteins are important for elucidating the various functions of SIRT1 and its role in stress resistance and survival in mammalian systems. In this study, we identified three novel interacting-partners for SIRT1 using a yeast-two hybrid system and co-immunoprecipitation. Furthermore, we showed that, by interacting with these novel binding partners, SIRT1 modulates three independent signaling pathways, which can potentially affect cellular stress response and survival.

5.1 TLE1: A NOVEL BINDING PARTNER FOR SIRT1

TLE1 is one of the four TLE (Transducin-Like Enhancer of split) genes that encode human transcriptional repressors homologous to the *Drosophila* corepressor groucho [285]. TLE

proteins are temporally expressed in embryogenesis where they are involved in developmental processes including neurogenesis, somitogenesis (body patterning), osteogenesis and hematopoiesis [43,287,319]. Gro/TLE bind to a wide variety of transcription factors (including Hairy/HES, Runx, Lef/Tcf, and Nkx proteins) and regulate many signaling pathways such as Notch and Wnt signaling. Aberrant activities of some of these pathways are implicated in human cancers, such as Notch in Acute Lymphocytic Leukaemia [312], Wnt in colorectal cancer [20] and Runx in Acute Myeloid Leukaemia [197]. The inhibitory role of TLE1 in Wnt signaling and other cell fate determination signals is dependent on phosphorylation status and involves histone deacetylase activity [29,44,129,227]. In our study, we discovered that the human TLE1 interacts with the class III histone deacetylase, SIRT1. Data from our laboratory suggested that SIRT1 repressed NF- κ B activity. Interestingly, *Tetsuka et al.* demonstrated that TLE1 and the amino-terminal enhancer of split (AES), a shorter Groucho-related protein lacking the WD40 repeats, bind p65 subunit and inhibit NF- κ B transcriptional activity [293]. Since it was shown that inhibition of NF- κ B activity derepresses expression of the bone morphogenetic protein-4 gene, indicating a putative role of vertebrate NF- κ B in transcriptional repression, e.g [36], it was speculated that if NF- κ B acts as an active repressor in vertebrates, it is likely that there are additional repressor binding sites and the respective DNA-binding proteins. This was based on the fact that in *Drosophila*, recruitment of Groucho is obligatory but is not sufficient for Dorsal-mediated repression. The *Drosophila* NF- κ B (Dorsal)-mediated repression requires additional repression elements in the proximity of the Dorsal binding sites and the binding of other DNA-binding repressor proteins to these elements. Although, SIRT1 does not bind DNA directly, it binds and regulates a number of histone proteins and transcription factors. HES1 and Hairy are two such bHLH domain-containing DNA-binding transcription factors, that are associated and

regulated by both SIRT1 and TLE1 [78,79,237,288]. Also, SIRT1 and TLE1 both are known to bind histone proteins, e.g Histone 3 and 4 [235]. Thus, it was possible that SIRT1 could act as the additional repression element that helps the Gro/TLE1 mediated NF- κ B repression. We investigated if the interaction of SIRT1 and TLE1 was relevant to regulation of NF- κ B transcriptional activity. We found that both SIRT1 and TLE1 could repress TNF α induced NF- κ B activity individually, but when these proteins were coexpressed, the repression on NF- κ B activity was further enhanced. While our investigation was going on, another group [320] reported that SIRT1 represses NF- κ B by deacetylating and inhibiting the transactivation domain of p65. While we had observed that depletion of SIRT1 leads to a robust increase in TNF α induced NF- κ B activity, we had not seen any effect of inhibition of SIRT1's catalytic activity on the same. By using a catalytic mutant of SIRT1, we showed that inhibition of NF- κ B activity was well achieved regardless of the sanctity of the catalytic core domain of SIRT1. To confirm this result, we used a 270 a.a long amino-terminal fragment (1-270a.a) of SIRT1, which did not possess the catalytic domain, and demonstrated that the amino-terminal 270 a.a fragment of SIRT1 protein was sufficient to repress NF- κ B activity. Co-expression of TLE1 with either of these catalytic mutants of SIRT1, further enhanced the repression in the same way as it did for the wild-type SIRT1 protein, indicating that TLE1 cooperates with SIRT1 to mediate repression of NF- κ B transcriptional activity. The importance of the presence of both SIRT1 and TLE1 for repression of NF- κ B activity was further demonstrated in knock-down experiments, where we knocked-down either SIRT1 or TLE1 in HeLa cells by using shRNAi, and showed that depletion of one protein affects the NF- κ B repression mediated by the other. Thus, we demonstrated a novel SIRT1 associating protein, TLE1, which plays a critical role in SIRT1 mediated repression of NF- κ B signaling, potentially through a novel deacetylase-independent mechanism.

While mounting evidence has documented the widespread importance of Gro/TLE-mediated repression in different biological contexts, relatively little is known about the mechanisms underlying Gro/TLE-mediated transcriptional repression. Characterization of proteins that directly or indirectly associate with the Gro/TLE family of proteins is important for insights into the mechanisms of Gro/TLE-mediated repression. Gro-interacting proteins have been grouped into two categories: the first category comprises the sequence-specific transcription factors that are responsible for the direct recruitment of Gro/TLE proteins to the DNA, and the second category consists of proteins that functionally contribute to Gro/TLE-repression activity. Evidence show that the TLE1 homologs in lower organisms; *Drosophila* and yeast, bind with proteins, such as Rpd3[44], Sin3, RbAP48 [52] and Ssn6 [100,251,300], which act either as histone deacetylase or an adaptor between TLE1 and DNA binding complexes. Similarly, changes in the phosphorylation status of TLE1 proteins have been documented to correlate with its repressional activity [129], indicating the importance of post-translational modification by signaling pathways on TLE1 mediated repression. We propose that SIRT1 could potentially be a second category TLE1 regulator. SIRT1 may regulate TLE1 mediated repression, either through its deacetylase activity or by acting as a scaffold protein, thus functioning to facilitate TLE1's recruitment to histones, transcriptional machinery or to other proteins of the TLE1 co-repressor complex.

Groucho (Gro)/TLE transcriptional corepressors are involved in a variety of developmental mechanisms, including neuronal differentiation. In particular, Gro/TLE1 interacts with forkhead transcription factor brain factor 1 (BF-1; also termed FoxG1) to regulate neuronal differentiation during cerebral cortex development and represses transcription together with Gro/TLE1[318]. Interestingly, SIRT1 deacetylates the mammalian Forkhead transcription factors

to regulate a variety of cellular processes. Also, while it has been shown that SIRT1 plays a role in embryogenesis and developmental processes, the mechanism of SIRT1's action in the same is not fully understood. Given the well established role of TLE1 in developmental processes, the interaction between SIRT1-TLE1 could potentially be relevant to regulation of development and other processes affected by TLE corepressors. The potential broader impact of SIRT-TLE1 interaction in regulating different cellular processes can be elucidated by further investigation.

5.2 SIRT1: A NOVEL REGULATOR OF EUKARYOTIC INITIATION FACTOR 2-ALPHA PHOSPHORYLATION AND STRESS RESPONSE

SIRT1 is known to regulate cellular stress response by acting through a number of proteins which predominantly function as transcription factors, such as: p53, FOXO proteins, NF- κ B and E2F1. SIRT1's role in longevity is believed to be linked with its role in regulating cellular stress responses. We discovered that SIRT1 interacts with the eukaryotic translation initiation factor, eIF2 α . This is the first time that SIRT1 has been shown to interact with a protein involved in translation regulation, as oppose to most of its targets that regulate gene expression at the transcriptional level. Furthermore, eIF2 α is a translation regulatory protein which plays a critical role during cellular stress response. Given SIRT1's established role in protecting cells under stress conditions and our observation that SIRT1 associates with eIF2 α , we asked if SIRT1 mediated its functions during stress response by regulating translation. Since phosphorylation of eIF2 α is the critical step through which eIF2 α plays a role in stress response, we investigated if SIRT1 affects phosphorylation of eIF2 α under various stress conditions. Using SIRT1 knock-down cell lines, we showed that depletion of SIRT1 is directly correlated with the upregulation

of eIF2 α phosphorylation in response to various different stress conditions. Interestingly, the eIF2 α phosphorylation was much more significant in SIRT1 depleted cells and was more sustained when compared to wild-type cells. However, CHOP, a downstream effector of the stress response pathway was expressed much later in SIRT1 deficient cells compared to SIRT1 positive cells. Delayed expression of the downstream proteins in the stress response pathway can lead to impaired stress response, which in turn can cause lethality. Consistent with this, the SIRT1 depleted cells showed significantly more sensitivity to oxidative stress induced by two different agents; paraquat and H₂O₂. Since SIRT1 is also known to protect cells from oxidative stress by regulating the Forkhead transcription factors, we examined if the increased sensitivity of the SIRT1 null cells in response to oxidative stress was due to blocked translation caused by delayed CHOP expression resulting in consistently upregulated eIF2 α phosphorylation. By using a translation inhibitory drug, we show that indeed the higher survival of the SIRT1 positive cells in response to oxidative stress was potentially due to recovery of translation in later phase of stress response, as inhibition of translation in wild-type cells affected their survival, making them similarly sensitive to the SIRT1 deficient cells.

Since phosphorylation of eIF2 α by specific kinases is critical to cellular stress response, we investigated if induction of stress or phosphorylation status of eIF2 α affects eIF2 α -SIRT1 association. Using two different stress treatments and mutant eIF2 α , that were either phosphorylation incompetent or phosphorylation mimetic, we demonstrated that SIRT1-eIF2 α association was not dependent on stress condition or eIF2 α phosphorylation. SIRT1, which is a deacetylase enzyme associated with eIF2 α and affected eIF2 α phosphorylation. Hence we further investigated if SIRT1's deacetylase activity plays a role in SIRT1- eIF2 α association. Our results demonstrated that SIRT1 associates with eIF2 α regardless of its catalytic activity.

Also, we could not detect eIF2 α acetylation under stress or no stress condition. These observations suggest that SIRT1 may associate with eIF2 α as a scaffold protein to regulate eIF2 α mediated stress response. Furthermore, SIRT1 may potentially be important with regards to the de-phosphorylation process of eIF2 α , rather than regulating eIF2 α phosphorylation. Further studies examining the impact of SIRT1- eIF2 α association with regards to the activity of the protein phosphatase1 (PP1) holoenzyme that dephosphorylates eIF2 α could provide important details about regulation of eIF2 α phosphorylation by SIRT1. Notably, in our studies regarding SIRT1-TLE1 interaction and its role in NF- κ B mediated transcription, we have demonstrated that SIRT1 may have a functional role independent of its catalytic activity. Although we do not rule out the possibility of SIRT1's deacetylase action to be involved in its regulation of eIF2 α phosphorylation or de-phosphorylation, the deacetylase independent functions of SIRT1 should also be kept in mind while investigating for the mechanism involved in SIRT1 mediated eIF2 α phosphorylation and stress response.

Genomic and proteomic profiling of cells have documented a lack of correlation between the mRNA level and protein levels of numerous genes [39,107,131,222,249], indicating that post-transcriptional and translational control may be more important for regulating gene expression. This may be especially relevant in situations where immediate and rapid responses are needed for survival, such as in pathogenic infection, chemical insult, nutrient deprivation and alike. SIRT1 has been documented to be a determinant of longevity in lower organisms and mammals. It is believed to function through sensing energy as well as regulating stress responses. Conceivably, SIRT1 may potentially regulate survival responses that involve immediate attention along with ones that are more constitutive. Our studies identified a novel binding partner for SIRT1: eIF2 α , a translation regulatory factor. Notably, SIRT1's association

with eIF2 α not only marks a new category of SIRT1 substrates, but also demonstrates that SIRT1 may help the survival processes by regulating gene expression at multiple steps in response to endogenous and exogenous stresses.

5.3 SIRT1: A REGULATOR OF THE TARGET OF RAPAMYCIN PATHWAY

The mammalian target of rapamycin (mTOR) regulates cell growth and metabolism in response to diverse environmental cues, such as growth factors, nutrients, energy and stress. In the past 5 years, growing body of evidence has suggested a role of mTOR in longevity. Partial inhibition of TOR function has been shown to result in significant increase in lifespan in yeast, worms and flies [141,150,151,303]. Although, the mechanism of TOR's action on lifespan is not known, it is speculated that inhibition of mTOR increases lifespan by mimicking calorie restriction [202]. Incidentally, SIRT1 is known to sense energy, nutrients and stress status of cells, thereby regulating metabolism and stress response. Furthermore, SIRT1 has been shown to mediate lifespan extension due to calorie restriction in yeast, worms, flies and potentially mice. The coincidental involvement of SIRT1 and mTOR in regulating metabolism, stress and energy responses, longevity, and age related diseases, hints towards a possible link between the functions and/or activities of these two proteins in regulating various cellular processes.

We demonstrated here that SIRT1 and mTOR are indeed interconnected, whereby SIRT1 negatively regulates mTOR signaling. Investigating an initial observation in our laboratory, that SIRT1 knock-out mouse embryonic fibroblasts show larger cell morphology compared to wild-type cells, we sought after the pathway that is widely known to be responsible for regulating cell growth and size, namely the mTOR signaling pathway. We discovered that SIRT1 depletion in

mouse tissue, and human and mouse cells resulted in higher mTOR activity, as shown by phosphorylation of the well characterized mTOR substrates: S6K, S6 and 4EBP1. Since growth factor, amino acid stress and hypoxia have been documented to downregulate mTOR activity, we tested if these stress conditions affected mTOR signaling in SIRT1 depleted cells. Our results showed that mTOR signaling is upregulated in SIRT1 depleted cells despite of stress conditions, indicating the importance of SIRT1 in downregulation of mTOR signaling in response to unfavorable conditions. Notably, SIRT1 depletion did not result in alteration of the expression levels of the proteins involved in mTOR signaling, such as mTOR, TSC1, TSC2, Rheb or Raptor. Using an mTOR complex-1 specific inhibitor rapamycin, we further demonstrated that the regulation of mTOR signaling by SIRT1 is upstream to the mTOR-complex-1, as rapamycin could successfully abrogate mTOR signaling in both wild-type and SIRT1 depleted cells. Also, we demonstrated that SIRT1's catalytic activity was important in regulating mTOR activity. We showed that the SIRT1 activator, resveratrol, inhibited mTOR signaling in a SIRT1-dependent manner, whereas treatment of control SIRT1 positive cells with SIRT1 inhibitor, nicotinamide, resulted in upregulated mTOR activity, similar to that observed in SIRT1 depleted cells.

TSC1-TSC2 complex is the most prominent upstream inhibitor of mTOR signaling, which integrates several upstream signaling cues, such as growth factor, energy, stress and possibly amino acids. In response to specific stimuli, such as hormones, low energy, low nutrient or hypoxia, specific kinases and regulatory proteins, such as Akt, AMPK and REDD, activate or inhibit the TSC2 protein of the TSC1-TSC2 complex, thereby regulating mTOR signaling. Hence, we investigated if SIRT1's inhibitory role on mTOR signaling was mediated through the main mTOR inhibitory complex, TSC1-TSC2. Using SIRT1 activator and inhibitor in TSC2 knock-out cells, we demonstrated that these chemicals could not regulate mTOR signaling in the

absence of TSC2, indicating that SIRT1 indeed requires TSC2 for down-regulation of mTOR signaling. Given our observations that SIRT1 acted upstream of mTORC1 and needed TSC2 for its action, we suspected that SIRT1 may associate with the TSC1-TSC2 complex to regulate mTOR signaling. Consistent with this, we finally demonstrated that SIRT1 associates with TSC2 in human cells. We further attempted to examine if TSC2 is an acetylated protein that is regulated by SIRT1's catalytic activity, but failed to detect acetylation of TSC2 under stress or growth condition. Even though we could not detect acetylation of TSC2, our results do not rule out the possibility that SIRT1 may potentially regulate other proteins of the mTOR regulatory complexes, in order to mediate its effect.

Further studies on specific signals that modulate TSC2 activity can give critical information with regards to SIRT1 mediated regulation of mTOR signaling. While regulation of TSC2 in response to growth factor/hormones and energy stress is known to be regulated by Akt and AMPK respectively, it is not yet known as to how REDD regulates TSC2 in response to oxidative stress. It has been documented that REDD1 acts downstream to Akt and upstream of TSC1/2 complex to downregulate mTOR signaling in an LKB independent manner. Given that SIRT1 is known to regulate oxidative stress response by regulating the forkhead transcription factor and our finding that it potentially regulates cellular stress response by regulating translation (Chapter 3), investigating the potential role of SIRT1 in REDD1 mediated downregulation of mTOR signaling could be very relevant. Interestingly, AMPK, which phosphorylates and activates TSC2 in response to energy stress, is believed to activate SIRT1 through the activation of nicotinamide phosphoribosyltransferase (Nampt), a NAD⁺ salvage pathway enzyme. Nampt overexpression or activation increases the [NAD⁺]/ [NADH] ratio, which in turn induces SIRT1 activity, thereby placing AMPK upstream to SIRT1. Although

REDD1 has been shown to act independent of LKB1-AMPK signaling to downregulate mTORC1, prolonged hypoxia can lead to energy depletion, thereby potentially interlinking the REDD1 and AMPK regulation on mTOR signaling. Since SIRT1 is well documented in the regulation of both energy and stress response, it will be potentially worthwhile to investigate if REDD1 mediates regulation of mTOR signaling in response to energy and/ or oxidative stress signals through SIRT1.

It was recently documented that eIF2 α phosphorylation leads to activation of PI3K/Akt/mTOR pathway. Although we detected upregulated eIF2 α phosphorylation and its downstream effects in SIRT1 depleted cells (as mentioned in Chapter 3), we did not observe measurable upregulation of Akt in SIRT1 depleted cells. Moreover, while looking for the upstream factor that SIRT1 was regulating to mediate mTOR signaling, we did not detect any measurable difference in the levels of AMPK activity either with regards to presence or absence of SIRT1. The above observation could be explained by our results suggesting that SIRT1 regulates mTOR signaling at the level of TSC2, which is downstream to both AMPK and Akt.

Given that SIRT1 and mTOR signaling are both implicated widely in age related diseases and longevity, our discovery of SIRT1 as a regulator of mTOR signaling is very relevant to the ongoing investigation with regards to elucidating the role of these two proteins in longevity. Notably, while inhibition of mTOR signaling increases longevity, activation of SIRT1 is important for lifespan extension. This is consistent with our observations that SIRT1 acts as a negative regulator of mTOR pathway. Further investigation on the impact of SIRT1 mediated regulation of mTOR signaling may open potential avenues in the field of aging research and related diseases such as neurodegenerative and metabolic disorders, as well as cancer.

5.4 CLOSING REMARKS

The presented study demonstrates interaction of the human sirtuin protein, SIRT1, with three novel binding partners: TLE1, eIF2 α and TSC2. While TLE1-SIRT1 interaction was demonstrated to be important for NF- κ B mediated gene transcription, the other two SIRT1 interacting proteins, eIF2 α and TSC2, play a role in translation regulation. This latter fact provides compelling evidence for a novel aspect of the function of SIRT1: its role in translation regulation.

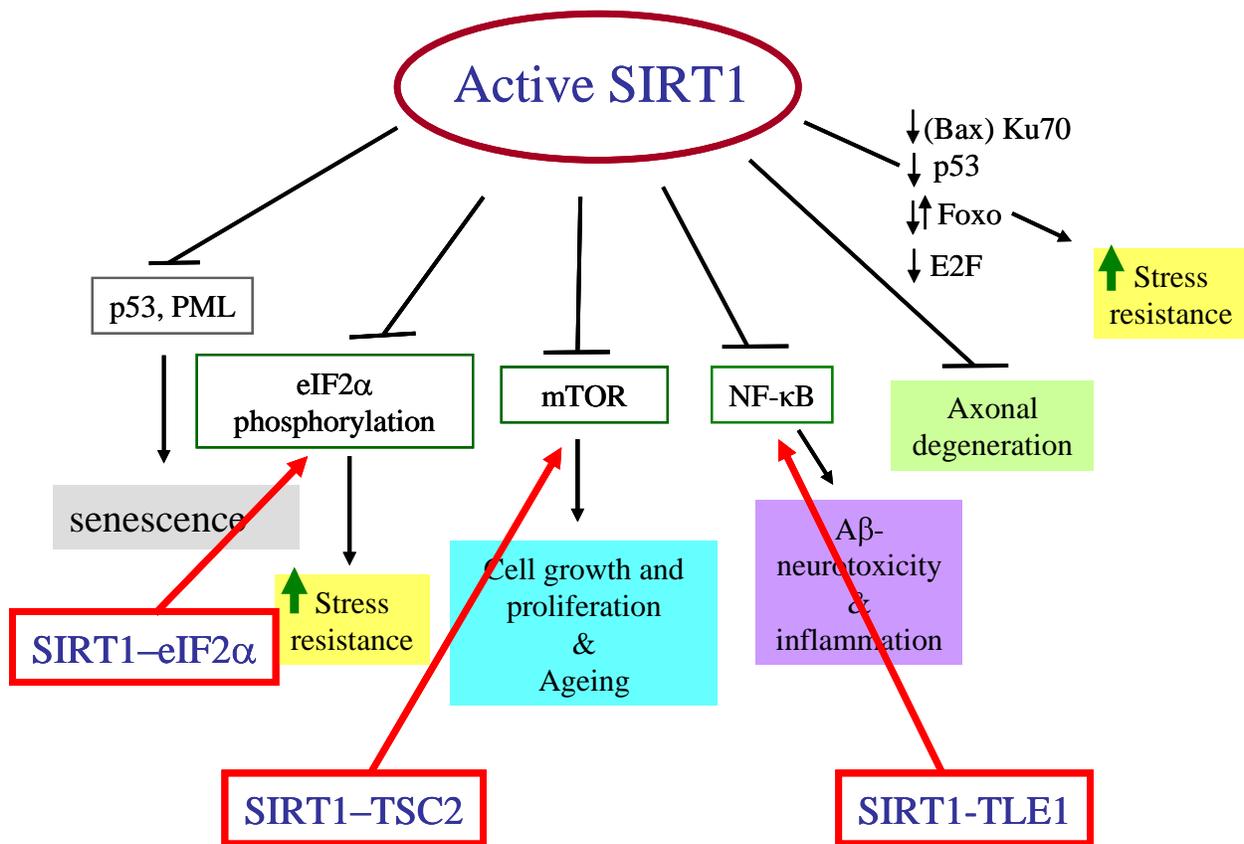


Figure 5.1 Three novel aspects of SIRT1 function in regulating cellular stress response and aging

SIRT1 affects cellular senescence by regulating p53 and PML. SIRT1 improves cellular resistance to stress by acting through the stress responsive proteins; the forkhead transcription factors (FOXO), E2F, p53, and Ku70. SIRT1 also protects against neuronal toxicity and degeneration. Here we show that SIRT1 regulates cellular stress

response by three additional and novel mechanisms, by interacting with TLE1, eIF2 α and TSC2 and regulating the pathways controlled by these proteins.

SIRT1 has been implicated in various processes that play a role during cellular stress. Our results have demonstrated three novel regulatory mechanisms that are independently affected by SIRT1 to potentially impact cellular stress response and survival. We showed that SIRT1 regulates NF- κ B, an important regulator of immune response and inflammation, and eIF2 α , a critical regulator of stress response, thereby, directly playing a role in cellular stress response. The mTOR pathway is a critical regulator of cell growth and proliferation in response to growth or stress signals, thereby playing an important role in cancer. Hence, by regulating the mTOR pathway, SIRT1 plays a role in cellular growth in response to stress thereby, potentially regulating cancer. This thesis thus demonstrates, for the first time, the direct regulation of these pathways by SIRT1, and lays grounds for a better understanding of SIRT1's role in cancer and aging.

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