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ABROL, Meena

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

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M. W. McBurney

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

P. Liston

D. Picketts

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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THE ROLE OF SIR2α IN MYOGENESIS

By

Meena Abrol

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Abstract

Protein acetylation is becoming recognized as an important modification in the regulation of many cellular pathways. The silent information regulator 2 (Sir2) proteins are a family of NAD-dependent protein deacetylases. Sir2 protein function in yeast has extensively been characterized as an essential mediator of gene silencing that occurs at the mating type loci, the telomeres and the rDNA regions. There are Sir2 homologues from yeast to mammals, but little is known about the role of Sir2 in the mammalian system. There is evidence that myogenesis is regulated by the acetylation state of the histones in the promotor region of muscle-specific genes as well as the myogenic transcription factors. MyoD is a myogenic transcription factor that plays a key role in myogenesis. Its activity is required to initiate and maintain transcription of muscle-specific genes. In addition to this, acetylation of MyoD is required for its activity. We set out to determine if MyoD is a target for deacetylation by Sir2α, and thus determine if Sir2α has a role in myogenesis. We determined that MyoD transactivation activity is not inhibited by Sir2α deacetylation. Over-expression of Sir2α-wt or a catalytically inactive mutant Sir2α-H355Y in C2C12 cells also showed that Sir2α deacetylase activity does not effect myogenesis. In addition to this, nicotinamide, a Sir2 inhibitor, had no effect on myogenesis while TSA significantly inhibited myogenesis in the C2C12 cells, suggesting that Class I and II HDACs have a more important role than Sir2 in regulating MyoD activity.

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
CaMK	calcium/calmodulin-dependent kinase
CAT	chloramphenicol acetyltransferase
cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ES cells	embryonic stem cells
GFP	green fluorescent protein
HATs	histone acetyltransferases
HDACs	histone deacetylases
Hda1p	histone deacetylase 1 protein
hSIRT1	human SIRT1
MCK	muscle creatine kinase
MEF2	myocyte-specific enhancer factor 2
MHC	myosin heavy chain
MyoD	myogenic determination factor
NAD	nicotinamide adenine dinucleotide
NES	nuclear export signal
NIA	nicotinamide
NLS	nuclear localization signal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pgk	phosphoglycerate kinase
pRb	retinoblastoma protein
Puro	puromycin
rDNA	ribosomal deoxyribonucleic acid
RLB	reporter lysis buffer
SDS	sodium dodecyl sulfate
ShPBS	stockholm phosphate buffered saline
Sir2α	silent information regulator 2 alpha
SIRTs	sir2 like proteins (sirtuins)
TBST	tris-buffered saline with tween
TE	tris-EDTA
TLC	thin layer chromatography
TSA	trichostatin A

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Chapter 1: Introduction

1.1 General Introduction

It is becoming clear that the post-translational modification of histone and non-histone proteins by acetylation is part of an important cellular signaling process controlling a wide variety of functions. The acetylation state of histone proteins plays a central role in gene regulation through changes of chromatin structure. In addition to this, the acetylation of many transcription factors is a key regulator of their function. Alterations in protein acetylation has been implicated in neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease [1-3], as well in various cancers, where histone deacetylase inhibitors are being tested in carcinoma cell lines and clinical trials as anticancer agents [4-6]. Sir2 protein function in yeast has extensively been characterized as an essential mediator of gene silencing, but its role in the mammalian system is unclear. In this study, we set out to determine if Sir2α, a mammalian NAD-dependent deacetylase, plays a role in regulating myogenesis by deacetylating the myogenic factor MyoD.

1.2 Chromatin Structure and Remodeling

Chromatin structure is a key component to the regulation of gene transcription. Both active and inactive genes are packaged into chromatin, where the nucleosome core particle plays a large role in DNA packaging. Each nucleosome consists of DNA associated with a histone octamer of histones H2A, H2B, H3, and H4, with variable

lengths of linker DNA between nucleosomes. Histone H1 is associated with the linker region of the DNA and appears to have a role in compacting nucleosomes into the higher order 10 and 30 nm chromatin filaments. However, a role for H1 in gene regulation remains controversial. The N terminal "tail" domains of histones protrude from the surface of the chromatin chains and provide an exposed surface for potential interactions with other proteins.

The purpose of chromatin remodeling proteins is to alter the nucleosomal structure so that genes are exposed to or hidden from transcriptional machinery. Chromatin remodeling occurs by non-covalent and covalent modifications of the chromatin structure. Non-covalent modifications involve the ATP-dependent chromatin remodeling complexes, which use the energy from ATP to expose nucleosomal DNA. These remodeling complexes are divided into three classes based on their homology in the ATPase domain and are: the SWI/SNF family, ISWI family and the Mi-2 family [7]. Out of these, the SWI/SNF family and ISWI family are the most well characterized, where they interact with a number of factors to exert their function. The mechanisms by which these enzymes modify the chromatin are not clearly understood, but two models exist. The first model involves the "sliding" of the DNA with respect to the nucleosome, where identical amounts of DNA moving in one direction along the nucleosome enters and exits the nucleosome package. This results in a nucleosome that is translationally repositioned and the DNA that was originally interacting with the histones becomes exposed. In the second model, conformational changes occur which create a high energy intermediate that collapses into several distinct remodeled nucleosomal states, some of which involve translational movement, and others that involve increasing the amount of

exposed DNA [8]. Experimental evidence indicates that the SWI/SNF family follows the conformational change mechanism while the ISWI family remodel chromatin based on the sliding model.

Covalent modifications occur at the histone tails, which are known to be modified by phosphorylation, acetylation, methylation, ubiquitination, and ADP-ribosylation. These modifications can affect histone-DNA and histone-histone interactions, and acetylation has been the most widely studied. Actively transcribed genes are in chromatin containing core histones that are extensively acetylated particularly on the N-terminal lysine residues of H3 and H4. The main acetylation sites include lysines 9, 14, 18, and 23 in H3, and lysines 5, 8, 12, and 16 in H4 [9]. Acetylation neutralizes the positively charged lysine residues of the histones, resulting in destabilization of the histone-histone and histone-DNA interactions. Histone acetylation is associated with actively transcribed chromatin, while deacetylated histones are associated with silent chromatin. The acetylation state of the chromatin is determined by the interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs).

1.3 Histone Deacetylases

1.3.1 Class I and Class II Histone Deacetylases (HDACs): The Classical HDAC family

HATs and HDACs are known to function in multiprotein complexes to regulate gene transcription, as well as the activity of transcription factors. HATs, such as CBP/p300, PCAF, and p160 family proteins, normally function as transcription activators, whereas HDACs function as transcription repressors. Three classes of HDACs exist to date and fall into two major families of proteins with HDAC activity. Members of the Class I and II fall into the classical HDAC family, while Class III are the Sir2 family.

The Class I HDACs have high homology to yeast Rpd3p, and include human HDACs 1, 2, 3, and 8. The yeast Rpd3p functions in a complex with Sin3 [10], Sap30 [11] and Ume6 [12] to repress chromatin. Ume6 is a zinc finger protein that binds URS1 elements in genes involved in meiosis and arginine catabolism. It is responsible for binding to Sin3 and recruiting the entire Rpd3p/Sin3/Sap30 complex to the target DNA region [10]. Sin3 is a transcriptional co-repressor that associates with Rpd3p to exert an inhibitory effect. Transcriptional repression by this complex is associated with deacetylation of all four core histones, with evidence of preferential deacetylation of histones H3 and H4. This histone deacetylation occurs over a limited range of up to two nucleosomes [10, 13].

In the mammalian system, the Class I human HDACs share high sequence homology. HDAC1 and HDAC2 are the most closely related to Rpd3. HDAC1, HDAC2

and HDAC8 are exclusively nuclear, since they lack a nuclear export signal (NES). HDAC3 has both a nuclear localization sequence (NLS) as well as a NES sequence, but is predominantly nuclear. These HDACs function by exerting their deacetylase activity in a number of pathways through interaction with specific protein complexes. Three protein complexes that have been characterized to contain both HDAC1 and HDAC2 are: Sin3 [14], NuRD [15], and Co-REST [16, 17].

HDAC1 and HDAC2 have been implicated to have a role in myogenesis, where MyoD-mediated transcriptional activity is inhibited in undifferentiated myoblasts by interactions between MyoD and HDAC1 [18]. In the initial stages of myoblast differentiation, there is a downregulation of HDAC1 protein and hypophosphorylation of the retinoblastoma protein (pRb). This hypophophorylation of pRb promotes the formation of a pRb-HDAC1 complex, which coincides with the dissociation of the MyoD-HDAC1 complex and transcription of muscle-specific genes [18]. It is thought that the pRb recruits HDAC1 to E2F, and thus inhibits E2F-dependent activation of genes involved in cell cycle progression [18].

The Class II HDACs are similar to yeast HdaIp, and include human HDACs 4, 5, 6, 7, 9, and 10. HdaIp is recruited to the DNA by the yeast TupI protein. TupI is a general repressor in yeast that interacts with Ssn6 to regulate many major physiological pathways such as the mating-specific genes STE2 and STE6 [19], and the membrane ATPase encoding gene ENA1 [20]. This corepressor complex is recruited to URS1 elements of target genes by sequence-specific DNA binding factors, such as α2, Mig1 and Crt1 [21]. Tup1 preferentially interacts with hypoacetylated histone H3 and H4 [13].

The Tup1-Ssn6 complex also functions with Rpd3 to repress genes that do not require Ume6 binding [21].

In the mammalian system, the Class II human HDACs have variable homology and function in a number of different pathways through nuclear-cytoplasmic shuttling in response to certain cellular signals. HDAC4, HDAC5 and HDAC7 share overall similarity, with a N-terminal NLS sequence, a C-terminal catalytic domain, and a NES sequence in the catalytic domain [22]. These HDACs have been extensively characterized for their role in myogenesis, where they interact with myocyte enhancer factor 2 (MEF2) to inhibit its activity [23]. HDAC4/5/7 are nuclear in an undifferentiated myoblast. Transport to the cytoplasmic depends on highly conserved serine residues that are phosphorylated by the calcium/calmodulin-dependent kinase (CaMK) [24, 25]. This leads to binding with the intracellular chaperone protein 14-3-3, which leads to CRM1 recognition of the NES sequence and export of the HDACs to the cytoplasm. HDAC 4 and 5 export liberates MEF2 and allows for activation of MyoD mediated myogenesis.

HDAC6 is unique because it contains two catalytic domains and is located exclusively in the cytoplasm [26]. It associates with microtubules and functions to deacetylate α-tubulin in assembled microtubules [26]. This deacetylation leads to decreased tubulin stability, which could be required to regulate microtubule-dependent cell motility. HDAC9 has been suggested to play a role in hematopoiesis, since it is highly expressed in cells of monocytic and lymphoid lineages and interacts with transcriptional repressors such as TEL and N-CoR, which have been implicated in the pathogenesis of hematological malignancies [27]. HDAC10 is the most recently discovered member of the Class II HDACs and a role for it has yet to be determined. It is

similar to HDAC6 in that it contains two catalytic domains, but this second domain is non-functional [28]. In addition to this, its transcript is subject to alternative splicing [29].

The expression patterns of Class I and II HDACs differ, where Class I HDACs are ubiquitiously expressed in most cell types and Class II HDACs are tissue-specific, being generally expressed in the heart, brain, kidney, skeletal muscle, thymus, placenta, lung, spleen and pancreas [22]. In general, Class I and II HDACs are sensitive to the fungal toxins trichostatin A (TSA) and trapoxin-B as well as sodium butyrate, which all inhibit their deacetylase activity. There are some exceptions, where HDAC6 is resistant to trapoxin-B and sodium butyrate as a result of its dual catalytic domain structure.

1.3.2 Class III HDACs: The Sir2 Protein Family

The Class III HDACs are a part of the silent information regulator (SIR) protein family, a family that has a pivotal role in yeast to silence three distinct chromosomal loci: silent mating type loci (HML/HMR), telomeric DNA regions, and ribosomal DNA (rDNA) repeats. Members of the silence information regulator (SIR) protein family have been shown to be associated in protein complexes at these chromosomal loci [30]. Sir2p plays a key role at these regions, since it functions in all of the regulatory complexes. It is the only SIR protein involved in rDNA silencing, is a histone deacetylase, and has weak ADP-ribose transferase activity. It is unique from the other classes of HDACs in that its deacetylase activity is dependent on NAD [31, 32] and is not inhibited by TSA or sodium butyrate. The yeast Sir2p is the only SIR protein that is universally conserved, with a

conserved core domain of 250 amino acid residues and with homologues present from yeast to humans [33].

In the yeast system, silencing at the HM loci and telomeric DNA regions is mediated via Sir2p, Sir3p, Sir4p, and Rap1. Rap1 binding sites have been characterized in both the HM loci and telomeric DNA regions, where Rap1 binding leads to the recruitment of Sir3p and Sir4p and subsequent binding of Sir2p [34]. Sir3p and Sir4p interact with the H3 and H4 histone tails to stabilize the silent chromatin structure and impede DNA transcription. Sir2p keeps the histone tails in a deacetylated state. At the rDNA regions, Sir2p functions in a complex with Net1p and Cdc14 to repress recombination within the rDNA clusters. Recombination can lead to excision of rDNA fragments in the form of extrachromosomal rDNA circles, which is a major cause of aging in yeast [35].

The NAD-dependent protein deacetylase activity of the Sir2 family has only recently been characterized. A number of groups have shown that histone deacetylation by Sir2p was coupled to cleavage of a high energy bond between the ADP ribose moiety of NAD and the nicotinamide group. In this process, the acetyl group is transferred from the lysine residue of the histone tail to the liberated ADP ribose moiety, forming the novel product 1-O-acetyl-ADP-ribose and the other reaction products, nicotinamide and deacetylated histone [31, 32]. Nicotinamide can inhibit the deacetylase activity of the Sir2 family of proteins.

Our knowledge of Sir2p structure comes from cystallography studies with a Sir2 homolog from *A. fulgidius* (Sir2-Af1). Sir2-Af1 shares 24% homology with yeast Sir2p. It is important to note that Sir2-Af1p contains only the core domain, lacking the N and/or

C-terminal extensions found in many eukaryotic members of the Sir2 family. This is characteristic of Sir2p homologs from thermophilic organisms [36]. Two crystal structures of Sir2-Af1 complexed to NAD have been determined at 2.1Å and 2.4Å resolutions [37]. These structures show that the protein consists of 2 main domains, a large domain containing a Rossmann fold and a small domain containing a zinc binding motif. A Rossmann fold domain is commonly found in NAD(H)/NADP(H) binding proteins, which is consistent with the requirement of NAD for Sir2 deacetylase activity. The zinc binding motif consists of 3 antiparallel strands and contains 2 Cys-X-X-Cys motifs that are conserved across the Sir2 family. These two pairs of cysteines coordinate a single zinc ion. NAD binds in a pocket between these two domains [37].

1.3.3 Sir2 in the Mammalian System

The Sir2 family of proteins is quite remarkable because the protein sequence of the core enzymatic domain is conserved from yeast to mammals. Its NAD-dependent deacetylase activity is also conserved across the Sir2 family. Despite this conservation, very few studies to date have revealed functions of Sir2p in the mammalian system. Proteins homologous to the yeast Sir2p in other organisms have different nomenclature. The human Sir2 family includes the Sir2-like proteins (sirtuins) SIRTs 1-7, where SIRT1, is the closest mammalian homologue to the yeast Sir2 protein. Many of the human SIRT proteins have different localization and function when compared to the yeast Sir2p. SIRT2 has been recently characterized as a tubulin deacetylase, where it deacetylates lys40 of α-tubulin. It is predominantly cytoplasmic and colocalizes and interacts with HDAC6 [38]. SIRT3 is a NAD-dependent deacetylase localized to the mitochondria, due

to the N-terminal mitochondrial targeting signal in the protein [39]. The function of SIRT3 in the mitochondria remains to be determined. The SIRT4 and SIRT5 sequences strongly resemble prokaryotic sirtuins, such as the bacterial Sir2-like gene CobB [40], and may have similar function to them. The SIRT6 and SIRT7 sequences are more similar to each other than Sir2 [41], and their functions remain to be characterized.

The mouse Sir2 family includes Sir2α-γ, where the mouse Sir2α is the closest mammalian homologs to the yeast Sir2 protein. The focus of our work is with the mouse Sir2α protein. Like the yeast Sir2, Sir2α protein is localized to the nucleus. But the function of Sir2α is not clear. Sir2α null ES cells created in our laboratory do not have elevated levels of acetylated histones and do not ectopically express silent genes [42]. The Sir2α null mice are smaller than normal at birth, but most of them thrive and eventually reach the size of the wild-type animals. The null animals are sterile and have abnormal gametogenesis. The Sir2α null males have low mature sperm counts with virtually no sperm motility, while the females do not ovulate due to the absence of proper hormone stimulation [43]. These observations suggest that the mammalian Sir2α protein plays a different role from the yeast Sir2 protein.

1.4 Myogenesis

1.4.1 General Overview

Muscle differentiation is accompanied by fusion of mononucleated myoblasts to multinucleated myotubes, transcriptional activation of muscle specific genes, and repression of genes associated with cell proliferation. The overall process of myogenesis

is triggered by low serum conditions which down-regulates cyclins D1, E and A and up-regulates several cdk inhibitors, locking the cyclin-cdk complexes in an inactive form [44]. This allows for withdrawal from the cell cycle and activation of muscle specific gene expression. Skeletal muscle differentiation involves the activation of myogenic basic helix-loop-helix (bHLH) regulatory factors that heterodimerize with E proteins, bind to regulatory regions of genes and thus activate muscle specific gene expression. These factors are MyoD, myogenin, myf5, and MRF4, where MyoD and Myf5 are expressed continually in proliferating myoblasts. Myogenin and MRF4 are late markers of myogenesis and are expressed in terminally differentiating myotubes [45]. Activation of muscle specific gene expression by the myogenic factors is dependent on the association with the MEF2 family of MADS-domain proteins [23]. Myogenic and MEF2 factors establish positive feedback loops that amplify and maintain expression as myoblasts differentiate.

1.4.2 bHLH Proteins and MyoD

The bHLH class of proteins have been known to play a key role in cell determination, proliferation, and differentiation. They are regulators of myogenesis, cardiogenesis, neurogenesis and hematopoiesis, by acting as transcriptional activators or repressors of gene expression. Much is known about MyoD in the mammalian system, where MyoD is the first and most well characterized bHLH protein that behaves as a transcriptional activator. It is a nuclear protein with a consensus NLS sequence at its N-terminus, followed by an activation domain and two well characterized domains: the basic region, and the helix-loop-helix domain (HLH). The basic region mediates DNA

binding at E-box consensus sequences (CANNTG) and contains 3 regions of basic amino acid (regions B1, B2, and B3) residues which are highly conserved among species of MyoD genes [46]. Crystal structure analysis of MyoD has shown that each MyoD monomer contacts 10 positions on the DNA backbone, where these interacting residues play a role in the recognition of the DNA sequence, and specificity and activity of MyoD [46, 47]. Arg-111 is present in all known bHLH proteins, and had direct contact with the G of the E box. Ala-114 allows Arg-111 to be positioned in the major groove of the target DNA. This positioning is crucial for correct DNA binding of Arg-111 [46, 47]. Correct DNA binding by the basic region of MyoD is required to unmask the N-terminal activation domain. It is theorized that this unmasking strategy is used to control both the activity and specificity of bHLH transcription factors.

In addition to its DNA binding function, the basic region contains three amino acids residues: Ala-114, Thr-115, and Lys-124 that are required for muscle gene activation but not DNA binding. Replacing these residues at the equivalent positions in the basic region of other bHLH proteins, such as E12, confers upon the protein the ability to activate myogenesis without affecting DNA binding [48]. These residues are refered to as the myogenic code and are conserved in all known myogenic bHLH factors in species from Drosophila to humans [49].

The helix-loop-helix region is located directly 3' to the basic region in MyoD. The HLH domain of MyoD mediates its heterodimerization with both itself and other bHLH proteins such as E proteins. E proteins are a family of widely expressed bHLH proteins that form heterodimers with tissue-specific bHLH proteins to regulate different sets of genes in various tissues. In myoblasts, E proteins function as transcriptional

coactivators of MyoD [50]. They heterodimerize with the HLH domain of MyoD and subsequently bind DNA at the E-box sites. Dimerization can occur in the absence of DNA binding, but is required for MyoD activity.

1.4.3 MEF2 factors

The members of the myocytes enhancer factor-2 (MEF2) family of transcription factors associate with myogenic bHLH proteins in order to activate skeletal myogenesis. Most muscle-specific genes examined to date have been shown to contain MEF2 binding sites in their regulatory regions. The MEF2 factors contain two well conserved domains: a MADS-box, and a MEF2 domain. The MADS-box is named for the first four members of the family to be identified, MCM1 in yeast, AGAMOUS and DEFICIENS in plants, and serum response factor (SRF) in vertebrates [51]. It located at the N terminus of MEF2 factors and is required for DNA binding at the conserved A/T rich sequence: YTA $(A/T)_4$TAR [52]. Directly C-terminal to the MADS-box is a 27 amino acid domain known as the MEF2 domain, which is unique to the MEF2 factors [51]. The MEF2 domain mediates DNA binding affinity and interactions with transcriptional cofactors. The C-terminal region of MEF2 contains the transcriptional activation domain.

The vertebrate MEF2 factors are MEF2A, MEF2B, MEF2C, and MEF2D [23]. They each have several splice variants and share more than 85% homology in the MADS and MEF2 domains. MEF2C is the first to be expressed in the developing embryo. Late in embryogenesis, MEF2A, MEF2B, and MEF2D transcripts become expressed in a wide range of cell types, whereas MEF2C expression remains restricted primarily to the skeletal muscle, brain and spleen [51].

MEF2 cannot activate myogenesis alone, but along with myogenic bHLH proteins MEF2 can synergistically activate a number of genes such as muscle creatine kinase (MCK) and myogenin. In addition to this, MEF2 can only interact with the heterodimers formed between myogenic factors and E12, and not the individual proteins. Both the MADS and MEF2 domains are required for interaction with the heterodimer and the transcriptional activation domain is required in either MEF2 or the bHLH factor.

1.4.4 Role of Acetylation and Deacetylation in Myogenesis

The dueling action of HATs and HDACs is emerging as a central process in the regulation of myogenesis. Activation of muscle specific genes requires the activation of MRFs as well as chromatin remodeling. Acetylation of MyoD is required for its activation. HATs such as PCAF and p300/CBP stimulate MyoD-dependent transcription by acetylating MyoD [53, 54] at lysine residues 99, 102, and 104 [53] and by acetylating the nucleosomal histones surrounding E- box sites. MyoD acetylation results in its increased DNA binding ability, increased binding to E proteins and increased transcriptional activity [55]. Deacetylation by HDACs antagonizes the stimulatory effect of HATs. MyoD interacts directly with class I HDACs, such as HDAC1, where they are associated together in undifferentiated myoblasts. Overexpression of HDAC1 inhibits MyoD activation of E-box luciferase reporters [18].

A major model of myogenic regulation has emerged from this dueling action of HATs and HDACs. As previously stated, MyoD, heterodimerized with E protein, is bound to the E-box regions of muscle specific genes. MEF2 is bound in this regulatory region at conserved A/T rich sites. In an undifferentiated myoblast, HDAC1/2 and

HDAC4/5 are bound to the MyoD heterodimers and MEF2, respectively. These HDACs keep the surrounding DNA region in a deacetylated state. Upon reduction of cell serum levels, Rb is dephosphorylated and proceeds to inhibit the cell cycle progresser E2F by recruitment of HDAC1/2. HDAC4/5 is removed from the region by nuclear export accomplished by the calcium/calmodulin-dependent protein kinase (CaMK) signaling pathway.

1.5 Rationale

The Sir2 family of proteins are NAD-dependent deacetylases. The yeast Sir2 protein functions to silence the HM loci, the telomeric DNA regions, and the rDNA repeats. The Sir2 family of proteins are highly conserved from yeast to mammals, but a definitive role for Sir2 in the mammalian system remains to be determined. We have focused on the closest mouse homologue to the yeast Sir2 protein, Sir2α. A number of transcription factors require acetylation of key lysine residues to be active, making them potential targets for deacetylation by Sir2α.

In addition to this, HDACs and other Sir2 homologues in mammals have been discovered to regulate function of the same proteins. Such is the case with HDAC6 and SIRT2, which both function to deacetylate α-tubulin and may play similar roles in cell motility [26, 38]. The proto-oncogene BCL6 is also regulated by both HDAC-dependent and Sir2-dependent pathways [56]. This reveals that Sir2α can function beyond modifications restricted to histone proteins as observed in yeast, and may be modulator of protein activity in a number of pathways.

From our laboratory, past experiments with P19 embryonic carcinoma stem cells have demonstrated that P19 cells transfected with MyoD do not differentiate into muscle, while fibroblasts transfected with MyoD quickly differentiate into myotubes. A closer look at these cells has shown that P19 highly express Sir2α while fibroblasts do not. A possible explanation for this observation is that high Sir2α levels in the P19 cells may be preventing them from undergoing myogenesis.

In myogenesis, acetylation of key myogenic transcription factors as well as acetylation of sequence-specific DNA regions is required to initiate the differentiation process. Acetylation of MyoD is required to activate its transactivational activity. Deacetylation of MyoD is not clearly understood. It was recently discovered that MyoD and HDAC1 physically interact in vivo. HDAC1 has been shown to deacetylate MyoD in vitro, but its role in vivo remains to be determined [57]. This leaves the process of deacetylation of MyoD to be further characterized. These observations take together suggest that is possible Sir2α may function to deacetylate MyoD in addition to the HDACs activities present, making Sir2α a candidate for a role in myogenesis.

1.6 Hypothesis

We hypothesized that MyoD is a target for deacetylation by Sir2α. This deacetylation inhibits MyoD transactivational activity and negatively regulates myogenesis. One prediction would be that we could modulate MyoD function by experiments in which we change Sir2α activity.

1.7 Objectives

1. In order to test this hypothesis, we made use of a MyoD responsive- luciferase reporter system. In this system we tested both Sir2α wt and the catalytically inactive Sir2α H355Y in cotransfection experiments with MyoD and the 4-E box-luciferase reporter, where it was predicted that Sir2α wt would decrease the luciferase activity and the Sir2α H355Y would increase luciferase activity.
2. After looking at the specific interaction between Sir2α and MyoD, the effect of Sir2α on the process of myogenesis was studied in a more biological model with C2C12 myoblast cells. The role Sir2α deacetylase activity in the C2C12 cell differentiation process was tested by overexpression of both Sir2α wt and the catalytically inactive Sir2α H355Y in these cells. It was predicted that overexpression of Sir2α wt would inhibit C2C12 cells from differentiating into myotubes, while overexpression of Sir2α H355Y would increase the extent of differentiation. In addition to this, specific HDAC and Sir2 inhibitors were tested for repression/activation of myogenesis. TSA is a specific Class I and II HDAC inhibitor which is known to inhibit myogenesis. Nicotinamide is an inhibitor of the Class III HDACs, and it was predicted that if Sir2α follows our hypothesis, nicotinamide would increase the extent of myogenesis in the C2C12 cells.

Chapter 2: Materials and Methods

2.1 Equipment

The instruments used in the experiments include an eppindorf Centrifuge 5417R (Brinkmann Instruments), a Beckmann J2-21M/E Centrifuge, a Beckman DU 640 Spectrophotometer for measurement of absorbances, a BioRad Gene Pulser and Capacitance Extender for electroporations, a Fisher Sonic Dismembrator Model 300 with microtip preparation of protein lysates, an EG&G Berthold Lumat LB 9507 Luminometer for measurement of luciferase, a Leica MZFLIII Microscope and a Perkin-elmer LS-5 Fluorescence Spectrophotometer for GFP readings, a Kodak M35A X-OMAT Processor for developing films, a Molecular Dynamics Storm 860 for CAT analysis, a Zeiss Axioskop2 Microscope for immunofluorescence work, and a Packard Tri-Carb 1600TR Scintillation Counter for measurement of radioactivity.

2.2 Plasmid Construction and Preparations

All restriction enzymes were obtained from New England Biolabs, GibcoBRL, and MBI Fermentas. Plasmids were propagated in E.coli strain DH5α and were isolated by the sodium dodecyl sulfate (SDS)/alkaline lysis method [58] or by use of QIAGEN Maxi prep kit (QIAGEN) .

2.3 Cell Culture and Transfections

C2C12 cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum (Medicorp Inc.). The cells were maintained at 37°C and 5% CO_2 in a humidified incubator and were harvested at 50-70% confluency. Cells were induced to differentiate by reducing serum levels to 2% horse serum (GibcoBRL) and the media was replenished every 48 hrs. 293T cells were cultured in Alpha modified Eagle's medium (GibcoBRL) supplemented with 7.5% donor bovine serum (Medicorp Inc.) and 2.5% fetal calf serum. The cells were maintained at 37°C and 5% CO_2 in a humidified incubator and were harvested at 80-90% confluency. 293T cells were plated at a density of 0.5 x 10^6 cells/60 mm dish 1 day prior to transfection. O41 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were maintained at 37°C and 5% CO_2 in a humidified incubator and were harvested at 80-90% confluency. O41 cells were plated at a density of 0.4 x 10^6 cells/60mm dish 1 day prior to transfection. Transient transfections were carried out using Fugene-6 (Boehringer Mannheim) or Gene Juice (Novagen) according to the manufacturer's instructions. For the luciferase assay, the transfection mix consisted of 2 μg GFP, 2 μg Sir2wt/mut, 2 μg MyoD, and 2 μg 4RE-luciferase reporter. Cells were harvested for the assay 24 hrs post transfection. For the CAT assay, the transfection mix consisted of 1 μg GFP, 4 μg Sir2wt/mut, 0.2 μg p53, and 2 μg p21r-CAT reporter. The transfections were done in duplicate. Cells were harvested for the assay 48 hrs post transfection.

2.4 Electroporations

C2C12 cells were harvested on the day of the electroporation. 1.2 x 10^6 cells were suspended in 0.8 ml cytomix buffer (120 mM KCl; 0.15mM $CaCl_2$; 10mM K_2HPO_4/KH_2PO_4, pH 7.6; 25 mM Hepes, pH 7.6; 2 mM EGTA, pH 7.6; 5 mM MgCl2; pH adjusted with KOH) per reaction. 10 μg of pgkpurolacZ (KJ35), pgkpuropgkSir2wt (ML19E), and pgkpuropgkSir2mut (ML20E) constructs were each digested with Sal1 (60U) overnight at 37°C, precipitated using 100% ethanol/10% 3M sodium acetate, washed with 70% ethanol, and resuspended in TE. The DNA was placed in a cold cuvette and incubated with the cells for 10 min on ice. Cells were then electroporated at a setting of 450 V and 250 μFD. Cells were incubated for an additional 10 min on ice and subsequently plated equally in four 150 mm dish. Puromycin selection was added to the dishes 48 hrs post electroporation at 3 μg/mL media. Media was changed on day 4 post selection and colonies were pooled and collected between day 9-14. These cells were plated immediately for differentiation and immunofluorescence analysis.

2.5 Assays

2.5.1 CAT Assays

O41 cells in monolayer were washed twice in PBS and collected by scraping in 0.25 M Tris buffer pH 7.4 and subsequently lysed by three freeze-thaw cycles. Supernatant was collected after centrifugation at 12000 rpm and 4°C for 5 min. Samples were heated at 65°C for 10 min to inactivate endogenous acetylating enzymes. Supernatant was

collected after centrifugation at 12000 rpm and 4°C for 5 min. 30 µL of protein lysate was used per reaction and prepared using the method described by Molecular Probes with the following modifications. 30 µl of cell extract was mixed with 5 µl of FAST CAT (Molecular Probes) substrate and incubated for 5 min at 37°C. 5 µl of 9 mM acetyl CoA (Sigma) was added and the reaction was incubated for 2 hrs at 37°C. The reaction was stopped by the addition of 160 µl of ice cold ethyl acetate (BDH). Each sample was vortexed for 20 seconds and then centrifuged at 14000 rpm for 5 min. The top ethyl acetate was removed and transferred to a clean tube and evapourated, leaving the sample completely dry. The dry samples were redissolved in 25 µl of ethyl acetate and 5 µl of the final samples were blotted 1 µl at a time on a TLC plate (EM Science). The plate was placed in a sealed chamber with 85:15 (v/v) chloroform (BDH):methanol (EM Science) solution and prerun filter papers. The conversion of substrate was developed by use of the Phosphoimager with the Storm 860- Blue fluorescence/chemifluorescence filter and analysed the ImageQuant 5.2 program.

2.5.2 HDAC assays

His-tagged proteins were purified by the QIAGEN nickel-column purification system, protocol 15 from the QIAexpressionist protocol book, with the following modifications. Dishes were transfected in duplicate and each dish was lysed in 500 µl of lysis buffer (10 mM imidazole) with a total lysate volume of 1ml. Each sample was sonicated 6 times for 15 sec/interval with a 10 sec on ice cooling period between intervals. Cells were subsequently lysed by three freeze-thaw cycles. Samples were spun down and the protein

lysate was collected. The total lysate of 1 ml was incubated overnight at 4°C with 100 µl of washed nickel nitrilotriacetic acid (Ni-NTA) superflow agarose beads. The beads were washed three times with 1 ml wash buffer (20 mM imidazole) and the His-tagged proteins were subsequently eluted off the beads using two elutions of 100 µl elution buffer (250 mM imidazole). The purified proteins were then dialyzed to remove the imidazole. 200 µl of protein sample was secured in 12-14 kDa range dialysis tubing and placed in a beaker containing 1L of PBS and continuously stirred at 4°C. The PBS was replenished once, and the samples were allowed to dialyze overnight. The HDAC assays were carried out using the Upstate Histone Deacetylase Assay Kit (Upstate) with histone H4 peptide as the substrate. 50 µg of histone H4 peptide was labelled with 12.5 µCi of [^{3}H]-Acetyl-CoA (NEN) following the protocol outlined by Upstate. 8000 CPM of [^{3}H]-Acetyl-histone H4 peptide was incubated with 20 µg purified His-Ump, His-Sir2 wt or His-Sir2 H355Y mutant at room temperature overnight, after which the reaction was stopped. The reaction mixture was spun down and 100 µl of the supernatant was counted using a scintillation counter.

2.5.3 Luciferase Assays

293T cells were collected by scraping the dishes and lysed following the Promega protocol with the following modifications. Cells were initially scraped into 1 ml PBS and collected into a microcentrifuge tube. The cells were spun down and resuspended in 500 µl of RLB 1X lysis buffer (Promega). A single freeze-thaw was performed and samples were vortexed for 15 seconds and centrifuged at 12000 rpm for 15 seconds at room

temperature. The supernatant was collected and 10 µl of prepared lysate was used along with 100 µl of assay substrate (Promega) per reaction using the single-tube luminometer with injector. The transfection was done in duplicate and results are based on two identical experiments with standard error.

2.6 Immunofluorescence

Cells were plated in 35 mm tissue culture dishes with 0.15% gelatin (Fisher Scientific) coated coverslips. Cells were fixed in 4% paraformaldehyde (EM Science) solution for 20 min at room temperature (for Sir2, Desmin antibodies) or in –20°C methanol for 5 min at –20°C (for the MF20 antibody). Cells were then washed in phosphate-buffered saline (PBS) and incubated overnight at 4°C with the primary antibody. For immunodetection of the Sir2 protein, a 1:100 dilution of the serum purified rabbit anti-Sir2 polyclonal antibody generated in the lab was used [42, 43]; for desmin detection a 1:10 dilution of a commercial mouse anti-desmin monoclonal antibody (CHEMICON international) was used, and for myosin-heavy chain detection a 1:2 dilution of a hybridoma (MF20) antibody was used. Each coverslip was washed three times in 10mM Stockholm PBS (ShPBS) for 10 min and then incubated for 45 min with either a 1:400 dilution of the goat anti-rabbit immunoglobin G cy3-linked antibody (Jackson ImmunoResearch Laboratories Inc) or a 1:100 dilution of the goat anti-mouse immunoglobin G fitc-linked antibody (Jackson ImmunoResearch Laboratories Inc). All the antibodies were diluted in 0.3% triton-X100 (Sigma)/ShPBS solution. Double labeling was done with a mixture of the primary antibodies, while the secondary

antibodies were incubated separately, all at the above mentioned dilutions and incubation times. Coverslips were washed three times in ShPBS for 10 min between each antibody incubation, stained with 1:2000 dilution of Hoescht 33258 (Sigma) for 5min, briefly washed once in ShPBS, and mounted on slides for viewing with the Zeiss microscope.

2.7 Western Blotting

Cell were trypsinized, resuspended in ice-cold RIPA C buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 2 mM NaF, 1x Roche Protease Inhibitor Cocktail Pellet), and sonicated for 15 seconds at a 35% output. The supernatant was collected after centrifugation at 10000 rpm and 4°C. Protein was then quantified using the Bio-Rad protein assay kit (BioRaD) and equal amounts of protein from each sample (usually 15-20μg) were separated on 8 or 10% PAGE. Proteins were then electrophoretically transferred to Hybond-C Extra (Amersham Life Science). Blots were blocked in 5% skim milk powder in Tris-buffered saline with tween (TBST) for 1hr at room temperature or overnight at 4°C prior to incubation with the primary antibody. All antibody immunodetection was done in 5% skim milk: TBST solution. The primary antibody was incubated for 1 hr at room temperature, while the secondary antibody was incubated for 30 min at room temperature. Three 10 min washes with TBST were done after incubation with each antibody. For immunodetection of Sir2, a 1:1000 dilution of the serum purified rabbit anti-Sir2 polyclonal antibody generated in the lab was used; for tubulin detection a 1:2 dilution of a mouse hybridoma (E7) supernatant was used; and for RGS-His6 tag detection, a 1:2000 dilution of a commercial mouse anti-his antibody

(QIAGEN) was used. The secondary antibodies used were either the goat anti-mouse anti-IgG heavy and light chain (BioRad) or the anti-rabbit anti-IgG heavy and light chain (BioRad), both horse-radish peroxidase conjugated antibodies at a dilution of 1:5000 and 1:2000, respectively. The membrane was then incubated with the KPL LumiGLO chemiluminescent substrate system (Mandel) and subsequently exposed to Kodak Scientific Imaging film (NEN Life Science Products, Inc) and developed.

2.8 Deacetylase Inhibitors

HDAC and Sir2 inhibitors were tested during the differentiation of the C2C12 cells. Cells were plate at 0.1 x 10^6 in 60mm dishes 1 day prior to the experiment. Cells were induced to differentiate by reducing serum levels to 2% horse serum and the inhibitor was added at this time. The media was replenished every 48 hrs and contained 2% horse serum along with the inhibitor. Inhibitors were used at the following concentrations: TSA 20nM, Nicotinamide (NIA) 5mM, 20mM.

Chapter 3: Results

3.1 Cloning of the Full-length Sir2α cDNA

3.1.1 Eukaryotic expression vectors encoding the full-length Sir2α wt and Sir2α H355Y cDNAs

The first step in the strategy to test for Sir2α function was to create an expression vector driving Sir2α wt and the catalytically inactive Sir2α H355Y mutant. Previous work in the laboratory had generated a Sir2α cDNA that contained only the exons 3 to 9 and was missing the 5' end of the gene. The 5' end was generated by PCR and TA cloned into TOPO4. A XhoI site was created upstream of the first ATG site in the full-length Sir2α cDNA. Referring to the cloning strategy in Figure 1, this 5' end was ligated to the existing cDNA sequence and inserted into the Bluescript II SK cloning vector. The whole full-length Sir2α cDNA cassette was removed and inserted into the mammalian expression vector pPOP. pPOP contains the phosphoglycerate kinase (pgk) promotor along with the 3' pgk sequence and has been used extensively in our laboratory.
The Sir2α H355Y protein contains a histidine to tyrosine point mutation in the catalytic domain of Sir2α, which was previously created in the laboratory. This mutation has been shown to abolish yeast Sir2 deacetylase activity [59, 60]. The cDNA sequence containing a cytosine to thymidine mutation, which confers the histidine to tyrosine mutation in the protein, was replaced in the full-length Sir2α wt cDNA in Bluescript II SK. Sequencing was done to confirm that the mutation was present.

Figure 1: Strategy in construction of the mammalian expression vectors encoding Sir2α wt and Sir2α H355Y. The 5' end of Sir2α wt was generated by PCR and TA cloned into TOPO4. A XhoI site was created upstream of the first ATG. The construct ML18, which contains the existing Sir2α wt cDNA sequence in Bluescript II SK was KpnI/EcoRV digested and dephosporylated. The KpnI/EcoRV Sir2α fragment was ligated with the vector and is referred to as MA6. The construct MA6 was BamHI/HindIII digested and dephosphorylated. The KJ203 construct, containing the Sir2α H355Y cDNA with a pPOP backbone, was BamHI/HindIII digested to obtain the mutant sequence. This fragment was ligated with the MA6 digested vector and is referred to as MA7. The whole full-length Sir2α wt/H355Y cDNA cassette was removed by BssHI digestion of this vector followed by blunt-ending with Klenow. The cassette was inserted into the mammalian expression vector pPOP after vector digestion with SmaI and dephosphorylation.

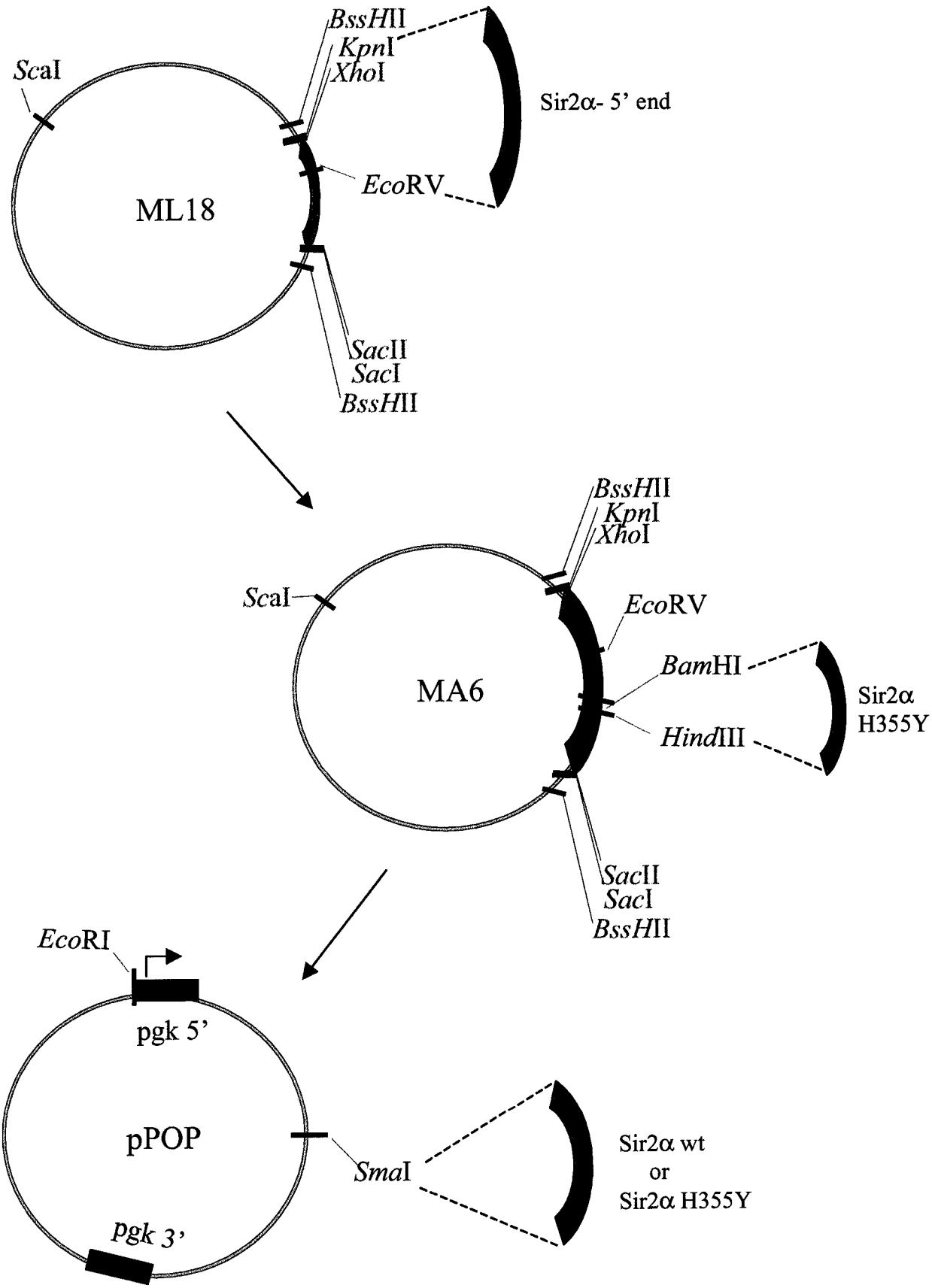

BssHII
KpnI
XhoI
ScaI
Sir2α- 5' end
EcoRV
ML18
SacII
SacI
BssHII
BssHII
KpnI
XhoI
ScaI
EcoRV
BamHI
Sir2α
H355Y
MA6
HindIII
SacII
SacI
BssHII
EcoRI
pgk 5'
pPOP
SmaI
Sir2α wt
or
Sir2α H355Y
pgk 3'

Figure 2: The mammalian expression vector encoding Sir2α wt. This construct consists of the Sir2α wt cDNA inserted downstream of the mouse pgk-1 promotor region and terminated by the pgk-1 polyadenylation site.

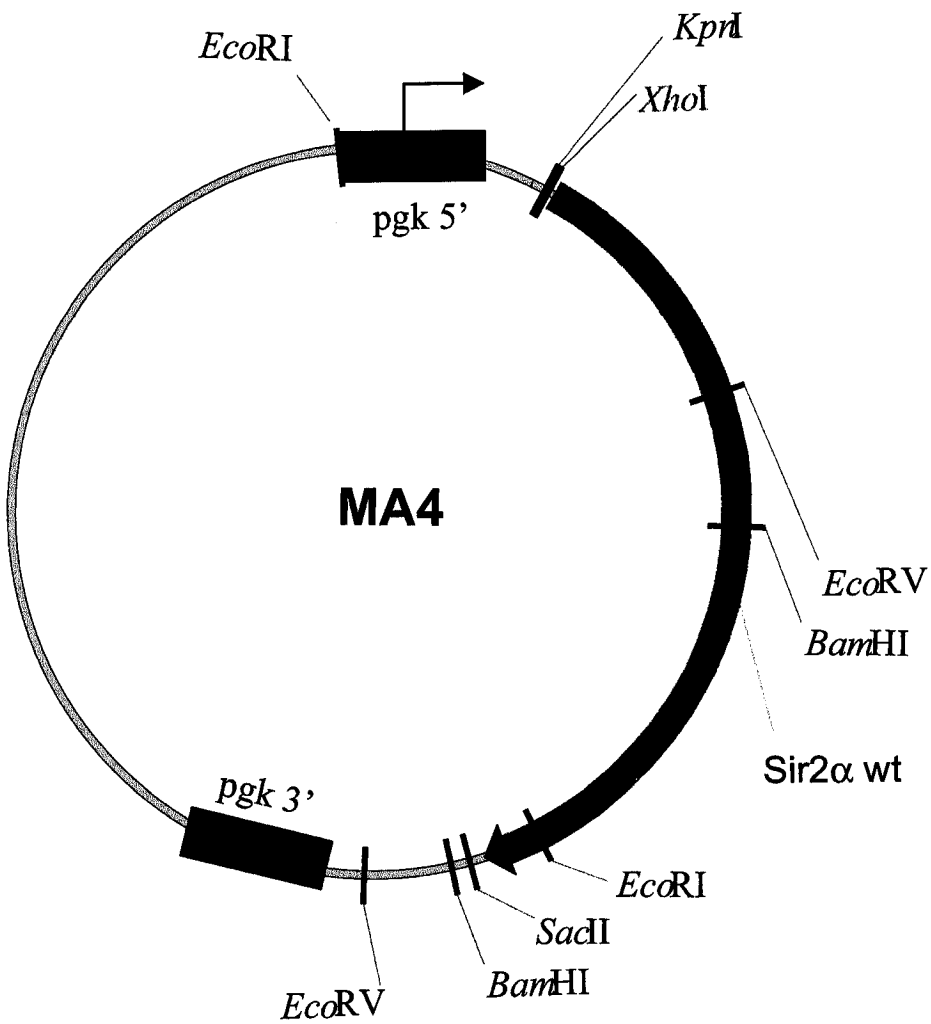
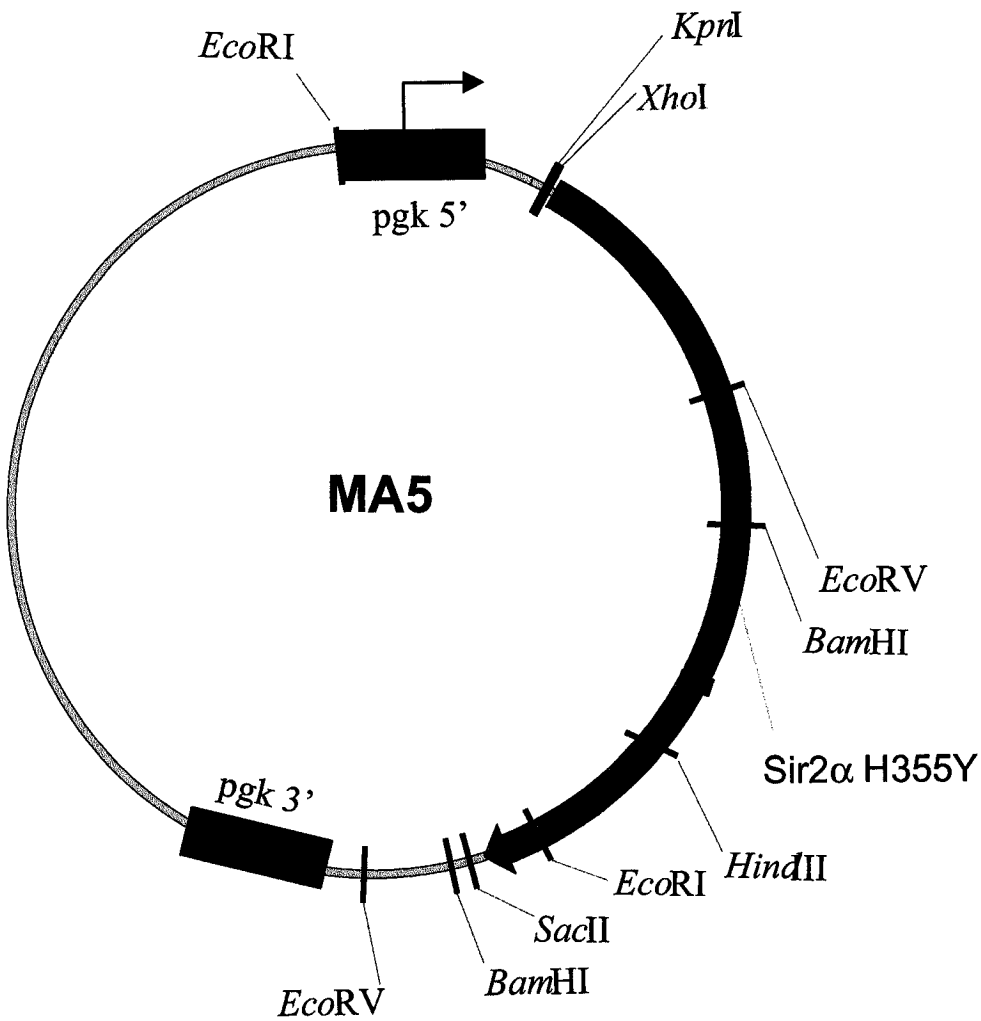

EcoRI
KpnI
XhoI
pgk 5'
MA4
EcoRV
BamHI
Sir2α wt
pgk 3'
EcoRI
SacII
BamHI
EcoRV

Figure 3: The mammalian expression vector encoding Sir2α H355Y. This construct consists of the Sir2α H355Y cDNA inserted downstream of the mouse pgk-1 promotor region and terminated by the pgk-1 polyadenylation site.


EcoRI
KpnI
XhoI
pgk 5'
MA5
EcoRV
BamHI
Sir2α H355Y
pgk 3'
HindIII
EcoRI
SacII
BamHI
EcoRV

The full-length Sir2α H355Y cassette was subsequently inserted into pPOP. These Sir2α wt and Sir2α H355Y constructs generated were referred to as MA4 and MA5 respectively (Figure 2 and 3).

We wanted to confirm that the MA4 and MA5 constructs could generate Sir2α protein. MA4 and MA5 were transfected in 293T cells, which are human kidney cells that express extremely low levels of the SIRT1 protein (homologous to yeast Sir2). This lowers the background of endogenous protein that could be picked up by the Sir2α antibody. The protein expression levels were detected via western blotting and immunofluorescence work. As shown in Figure 4, Sir2α wt and Sir2α H355Y mutant proteins were expressed robustly from the MA4 and MA5 constructs, respectively. However, all Sir2α detected in the cells migrated at a higher molecular weight of 120 kDa, rather than the predicted size of 80 kDa. This could be due to post-translational protein modifications. Immunofluorescence work showed that these Sir2α wt and Sir2α H355Y proteins were localized to the nucleus, like the endogenous Sir2α protein (Figure 5).

3.1.2 Eukaryotic expression vectors encoding the His tagged full-length Sir2α wt and Sir2α H355Y cDNAs

It was beneficial to tag these full-length Sir2α wt and H355Y mutant cDNA so that when transfected, they could be detected apart from the endogenous Sir2α protein. In addition to this, it was previously determined that our polyclonal Sir2α antibody was unable to pull down Sir2α in immunoprecipitation studies. Therefore, with the tagged

Figure 4: Sir2α protein expression levels from the Sir2α wt and Sir2α H355Y mammalian expression vectors. 293T cells were transfected with these constructs and protein expression levels were detected via western blotting at 24 hrs post transfection. Sir2 levels were detected with the rabbit polyclonal Sir2 antibody and tubulin was detected with the E7 mouse hybridoma. Sir2α protein was detected at 120 kDa and the transfected samples had much higher Sir2α protein levels (lanes 3 and 4) than the pgk-GFP transfected control (lane 5) or the untransfected an R1 and P19 cells (lanes 1 and 2, respectively).

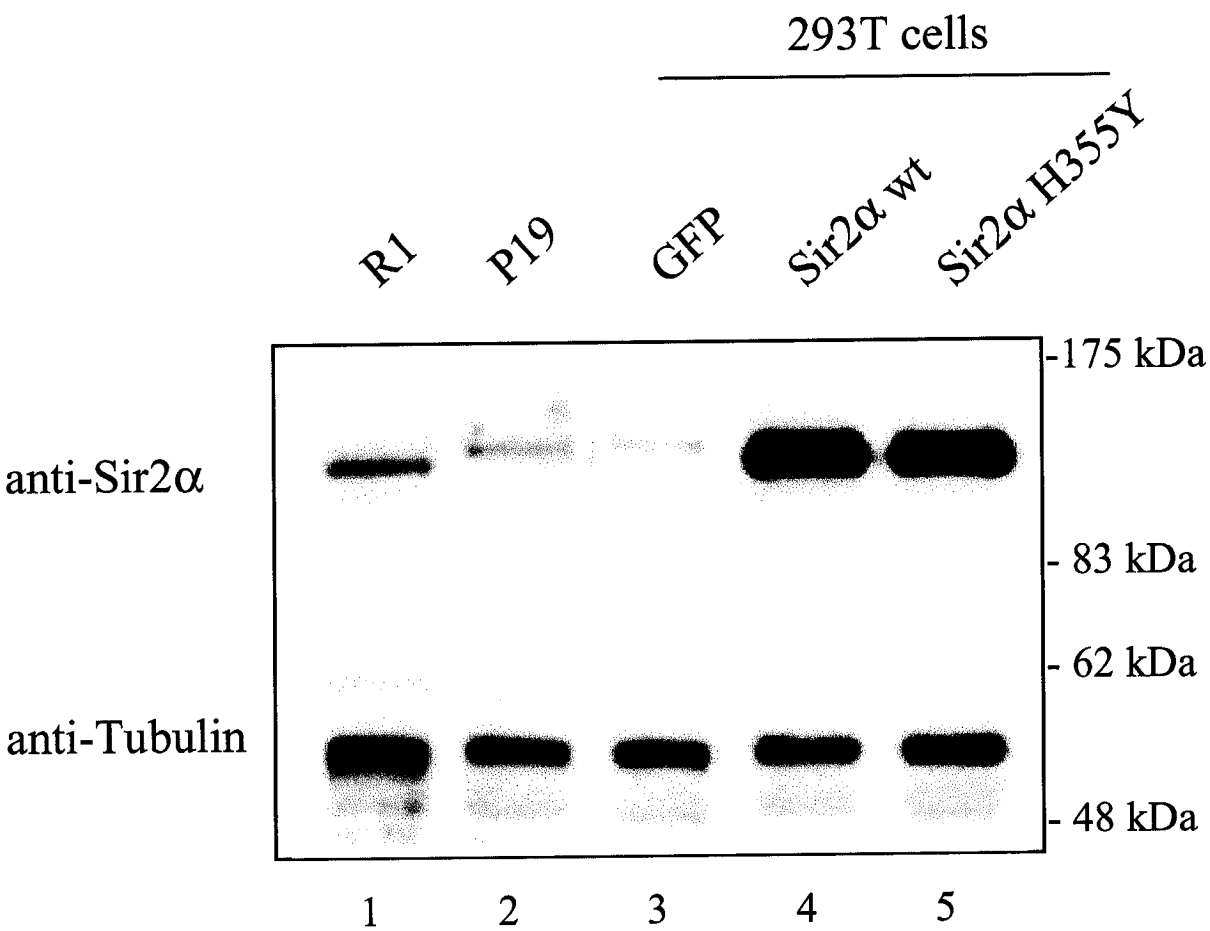
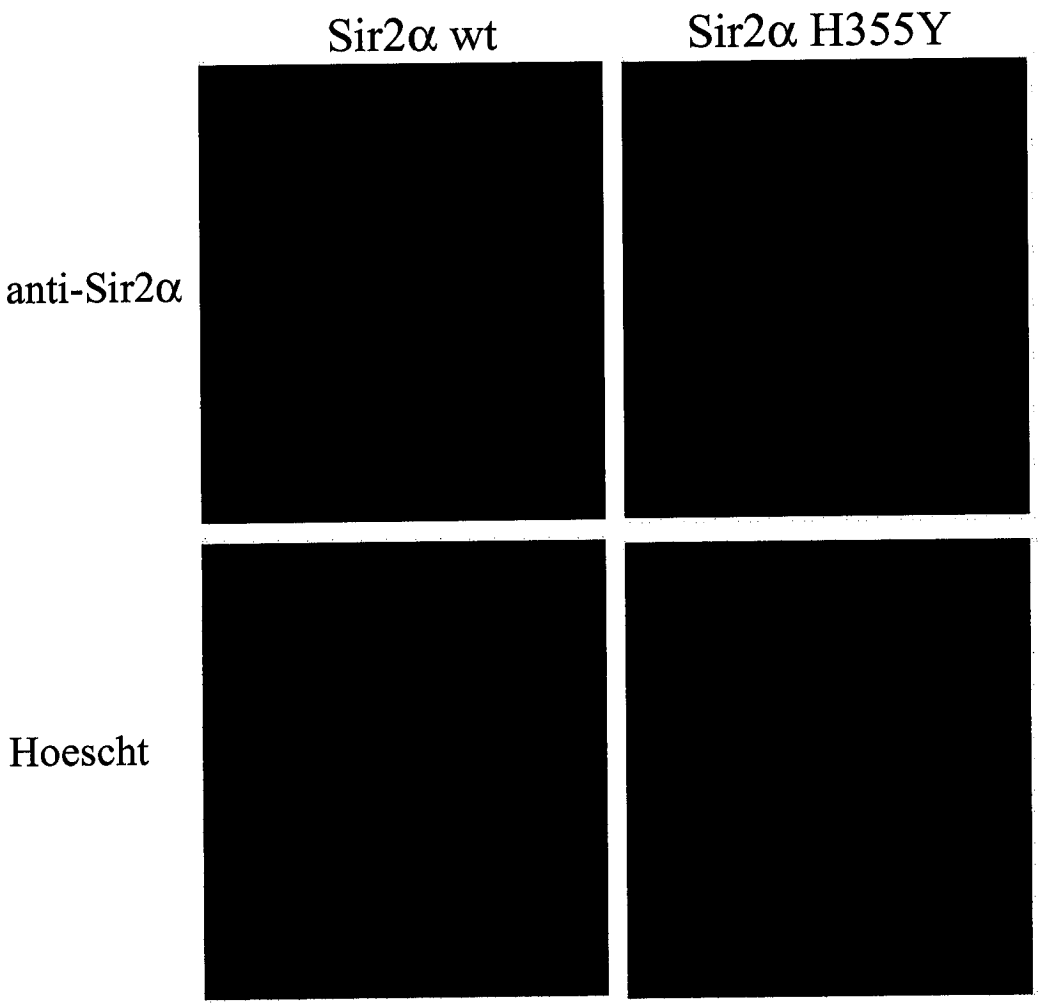

293T cells
R1
P19
GFP
Sir2α wt
Sir2α H355Y
anti-Sir2α
anti-Tubulin
-175 kDa
- 83 kDa
- 62 kDa
- 48 kDa
1
2
3
4
5

Figure 5: Cellular localization of the Sir2α protein expressed from Sir2α wt and Sir2α H355Y mammalian expression vectors. 293T cells were transfected with these constructs and the cellular localization of the Sir2α protein was detected by immunofluorescence at 24 hrs post transfection. The two upper panels show Sir2α staining via the rabbit polyclonal Sir2α antibody with indirect labeling with the Cy3-conjugated anti-rabbit IgG. The two lower panels show Hoescht staining of the same cells. Clear nuclear staining of both the Sir2α wt and Sir2α H355Y proteins can be seen.


Sir2α wt
Sir2α H355Y
anti-Sir2α
Hoescht

protein, the His antibody could be used in immunoprecipitation studies and the proteins could be purified by the nickel column system. By collaboration with Chris Kamel, an Arg-Gly-Ser- His_6 tag (RGS-His_6) was created from annealed oligonucleotides. This tag was attached directly upstream of the full-length Sir2α wt and Sir2α H355Y cDNAs (Figure 6 and 7) by use of a standard linker tailing method. Both the Sir2α wt and Sir2α H355Y His tagged proteins were detected by the His and Sir2α antibodies on a western blot (Figure 8). Immunofluorescence work revealed that both His-Sir2α wt and His-Sir2α H355Y proteins were localized to the nucleus in a similar pattern to the endogenous protein (Figure 9).

We concluded from this work that the Sir2α expression vectors created could generate protein that was detected by a specific Sir2α antibody to be localized to the nucleus. In addition to this, both the untagged and His- tagged Sir2α wt and Sir2α H355Y proteins generated could be detected by western blotting along with the endogenous protein, but the Sir2α protein in untransfected and transfected cells all migrated at a rate consistent with a molecular weight of 120 kDa rather than the predicted size of 80 kDa.

3.2 Enzymatic activity of the expression vectors encoding the Sir2α wt and Sir2α H355Y proteins

The expression vectors generated for Sir2α wt and Sir2α H355Y contained the consensus sequence for the Sir2α protein and could generate protein detected by

Figure 6: Construction of the mammalian expression vector encoding His-Sir2α wt. This construct consists of an Arg-Gly-Ser-His_6 tag (RGS-His) inserted upstream of the Sir2α wt cDNA sequence, flanked by both the pgk-1 promotor and polyadenylation regions respectively. MA4 was KpnI/XhoI digested and ligated with the annealed RGS-His_6 oligonucleotides. This expression vector was made in collaboration with Chris Kamel and is referred to as CK1.

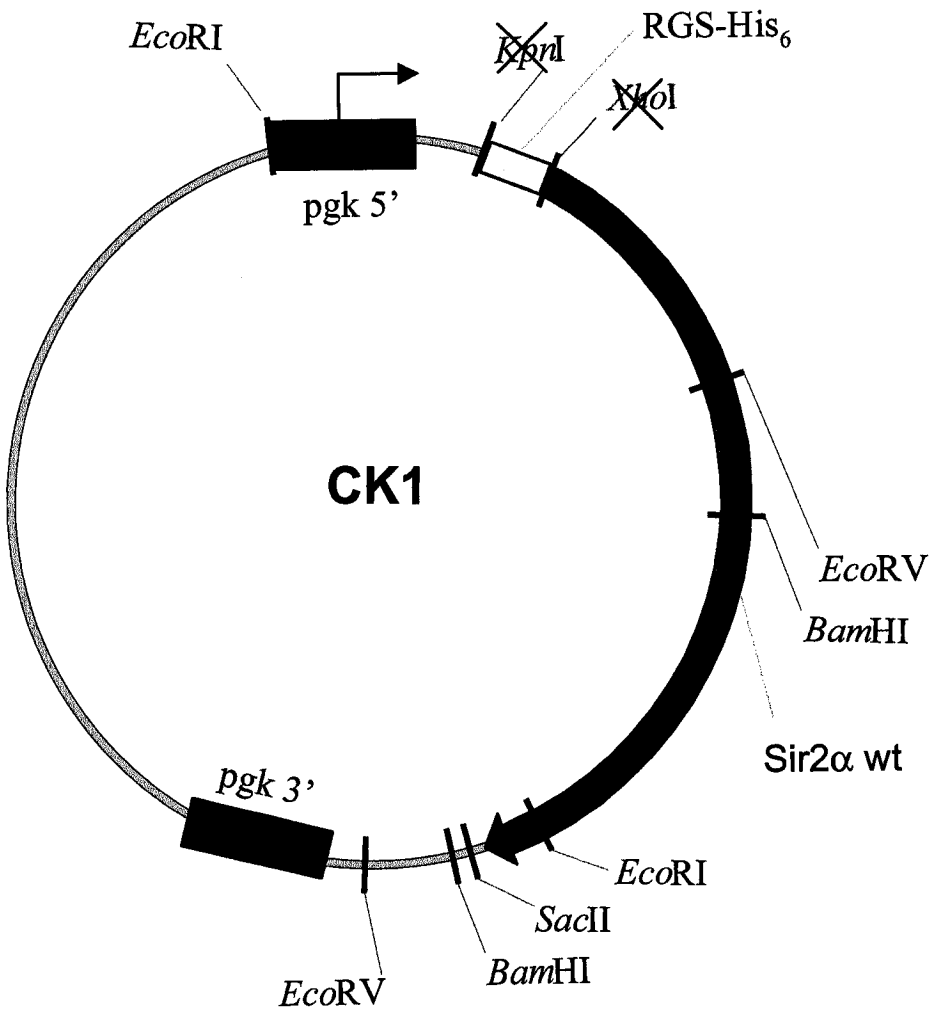

EcoRI
KpnI
RGS-His6
XhoI
pgk 5'
CK1
EcoRV
BamHI
Sir2α wt
pgk 3'
EcoRI
SacII
BamHI
EcoRV

Figure 7: Construction of the mammalian expression vector encoding His-Sir2α H355Y. This construct consists of a Arg-Gly-Ser-His_6 tag (RGS-His) inserted upstream of the Sir2α H355Y cDNA sequence, flanked by both the pgk-1 promotor and polyadenylation regions respectively. MA5 was KpnI/XhoI digested and ligated with the annealed RGS-His_6 oligonucleotides. This expression vector was made in collaboration with Chris Kamel and is referred to as CK2.

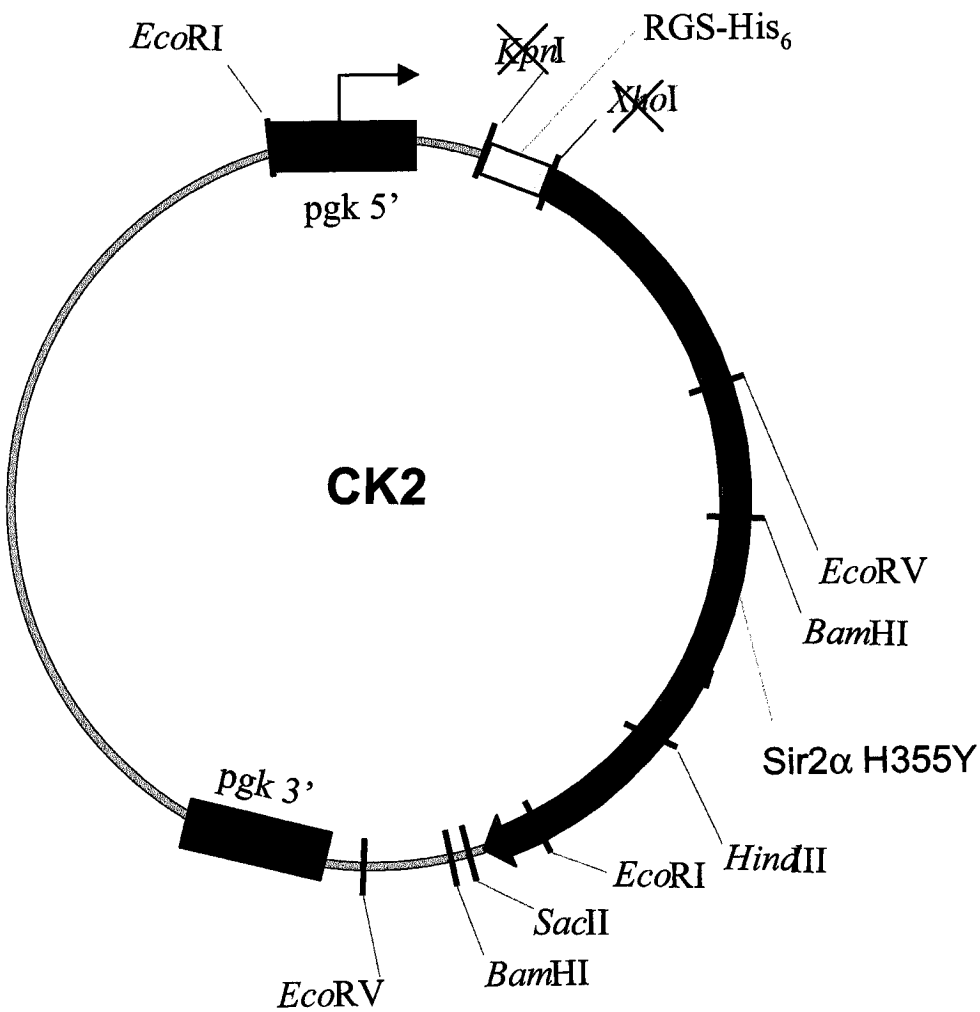

EcoRI
KpnI
RGS-His6
XhoI
pgk 5'
CK2
EcoRV
BamHI
Sir2α H355Y
pgk 3'
EcoRI
HindII
SacII
BamHI
EcoRV

Figure 8: Sir2α protein expression levels from the His-Sir2α wt and His-Sir2α H355Y mammalian expression vectors. 293T cells were transfected with the CK1 and CK2 constructs and protein expression levels were detected via western blotting at 24 hrs post transfection. Sir2α levels were detected with both the rabbit polyclonal Sir2α antibody and the mouse polyclonal His antibody, where Sir2α protein was detected at 120 kDa.

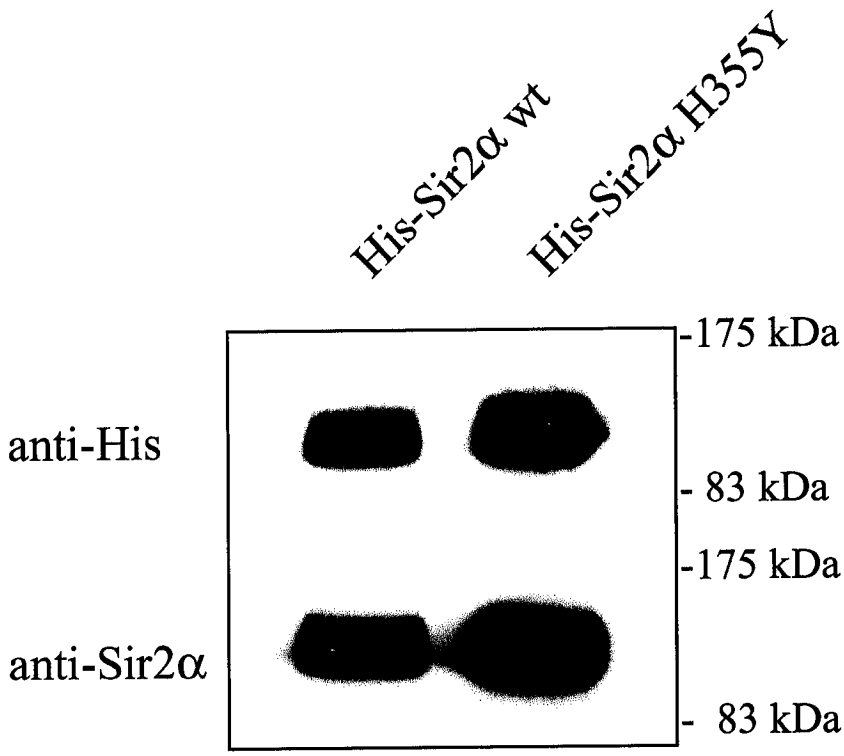
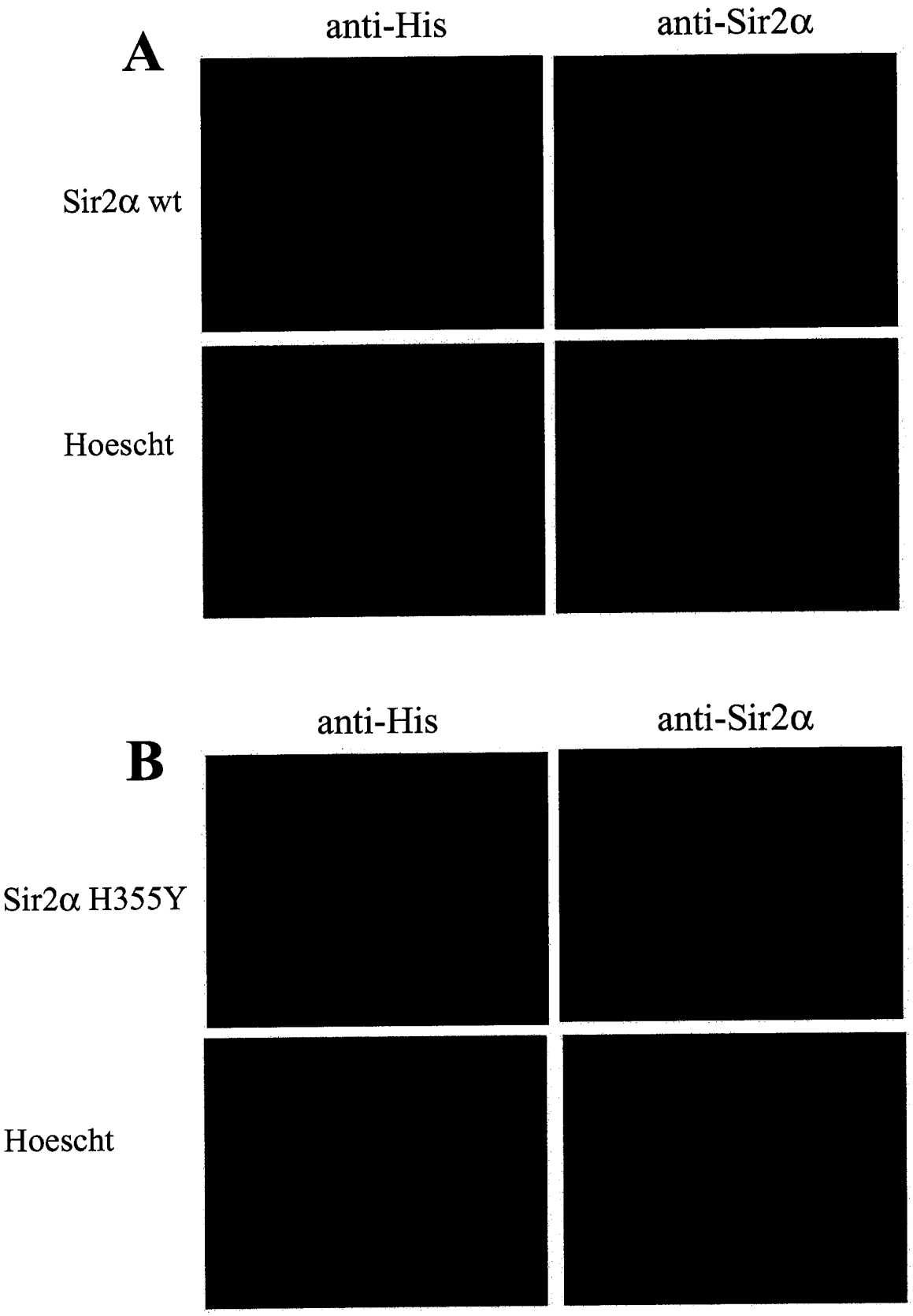

His-Sir2α wt
His-Sir2α H355Y
anti-His
anti-Sir2α
-175 kDa
- 83 kDa
-175 kDa
- 83 kDa

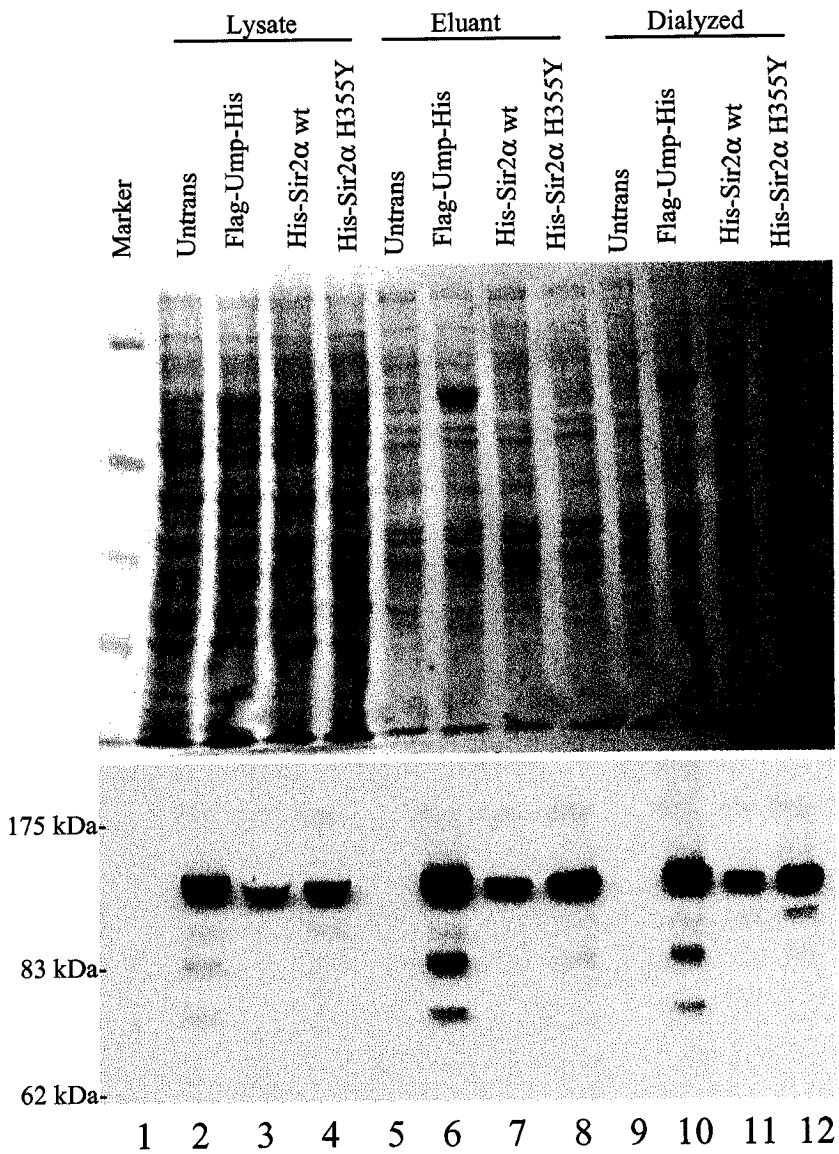
Figure 9: Cellular localization of the Sir2α protein expressed from the His-Sir2α wt and His-Sir2α H355Y mammalian expression vectors. 293T cells were transfected with the CK1 and CK2 constructs and the cellular localization of the Sir2 protein was detected by immunofluorescence at 24 hrs post transfection. Panel (A) shows staining of His-Sir2α wildtype while Panel (B) shows staining of His-Sir2α H355Y mutant. The upper two pictures of each panel show clear nuclear Sir2α staining via the mouse polyclonal His antibody or rabbit polyclonal Sir2α antibody with indirect labeling with the Cy3-conjugated anti-rabbit IgG. The lower two pictures of each panel show Hoescht staining of the same cells.


A
anti-His
anti-Sir2α
Sir2α wt
Hoescht
B
anti-His
anti-Sir2α
Sir2α H355Y
Hoescht

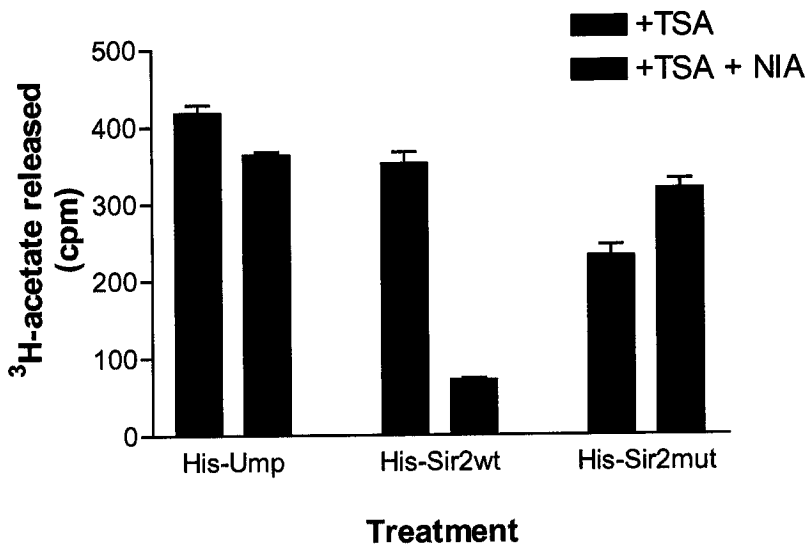
specific Sir2α antibody. By analogy with the Sir2 protein from yeast, we reasoned that the histidine to tyrosine substitution at residue 355 of Sir2α would result in a loss of deacetylase activity. In order to determine Sir2α enzymatic activity from our expression constructs, we carried out histone deacetylase assays. The yeast Sir2 protein is known to deacetylate histone proteins, and in this system [^{3}H]-acetyl-histone H4 peptide was used as a substrate. In order to carry out this assay, Sir2α protein was first purified from 293T cells transfected with either His-Sir2α wt or His-Sir2α H355Y. Flag-Ump-His was used as a positive control for the purification and a negative control for the assay. The His-tagged proteins were purified from the total cell lysates using the nickel column purification method, followed by dialyzing the proteins to remove imidazole. The proteins were successfully purified from total cell lysates, running at 120 kDa. Referring to Figure 10A, it is clear that the His-tagged protein from the total lysate (lanes 1-4) was purified into a more concentrated lysate (lanes 5-8), and there was minimal loss of protein from dialyzing (lanes 9-12). There was other bands picked up on the western blot probed with the anti-His antibody (Figure 10A, lower panel), which suggests that the purification process resulted in some degradation of the His-tagged proteins. These purified protein samples were then used in the deacetylation assay. Flag-Ump-His, His-Sir2α wt and His-Sir2α H355Y purified proteins were incubated with the [^{3}H]-acetyl-histone H4 peptide along with TSA and with/without nicotinamide and the release of [^{3}H]-acetyl-product was measured. Results show that the His-Sir2α wt protein had histone deacetylase activity that could be inhibited by nicotinamide (Figure 10B, 10C). The His-Sir2α H355Y protein did not possess nicotinamide inhibitable activity, where the histone deacetylase activity increased with the addition of nicotinamide (Figure 10B).

Figure 10: The histone deacetylase assay with the His-Sir2α wt and His-Sir2α H355Y purified proteins. 293T cells were transfected with Flag-Ump-His, His-Sir2α wt or His-Sir2α H355Y. Cells were harvested at 48 hrs post transfection and His-tagged protein was purified. (A) Protein expression levels were detected via amido black staining (upper panel) and western blotting (lower panel). The His-tagged proteins were detected in high concentration in the total lysate (lanes 1-4) and the purified proteins samples (lanes 9-12), and low levels of His-tagged protein remained unbound (lanes 5-8). The His-tagged proteins were detected using the mouse polyclonal His antibody, and all ran at approximately 120 kDa. (B) The His-tagged purified protein samples were used in a histone deacetylase assay, where the protein samples were incubated with a ^{3}H-acetyl-H4 peptide substrate in the presence of trichostatin A (TSA) with/without nicotinamide (NIA). ^{3}H-acetate released was inhibited in the nicotinamide (NIA) treated His-Sir2 wt purified extract, but not the His-Sir2 H355Y protein extract. (C) A plot of the Sir2-like activity observed from the results of the assay in B), where the difference between the with and without nicotinamide treatment gives the Sir2-like activity.

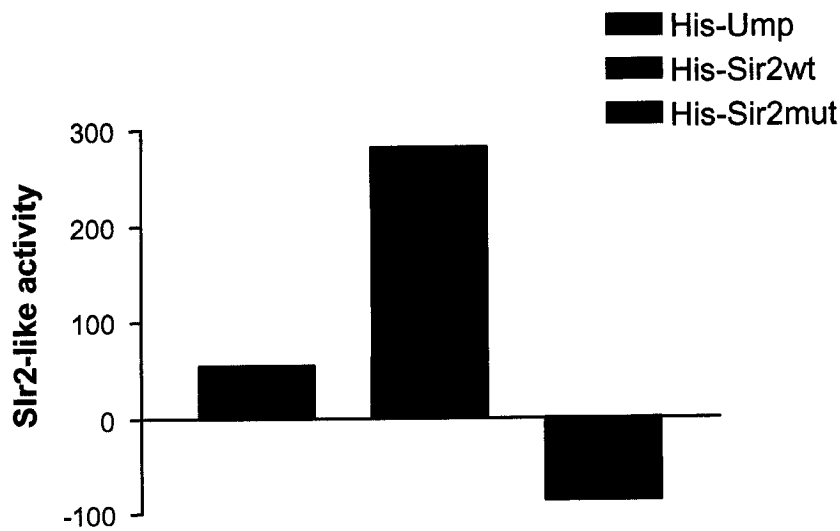
A


Lysate
Eluant
Dialyzed
Marker
Untrans
Flag-Ump-His
His-Sir2α wt
His-Sir2α H355Y
Untrans
Flag-Ump-His
His-Sir2α wt
His-Sir2α H355Y
Untrans
Flag-Ump-His
His-Sir2α wt
His-Sir2α H355Y
175 kDa-
83 kDa-
62 kDa-
1 2 3 4 5 6 7 8 9 10 11 12

B


+TSA
+TSA + NIA
500
400
300
200
100
0
^{3}H-acetate released (cpm)
His-Ump
His-Sir2wt
His-Sir2mut
Treatment

C

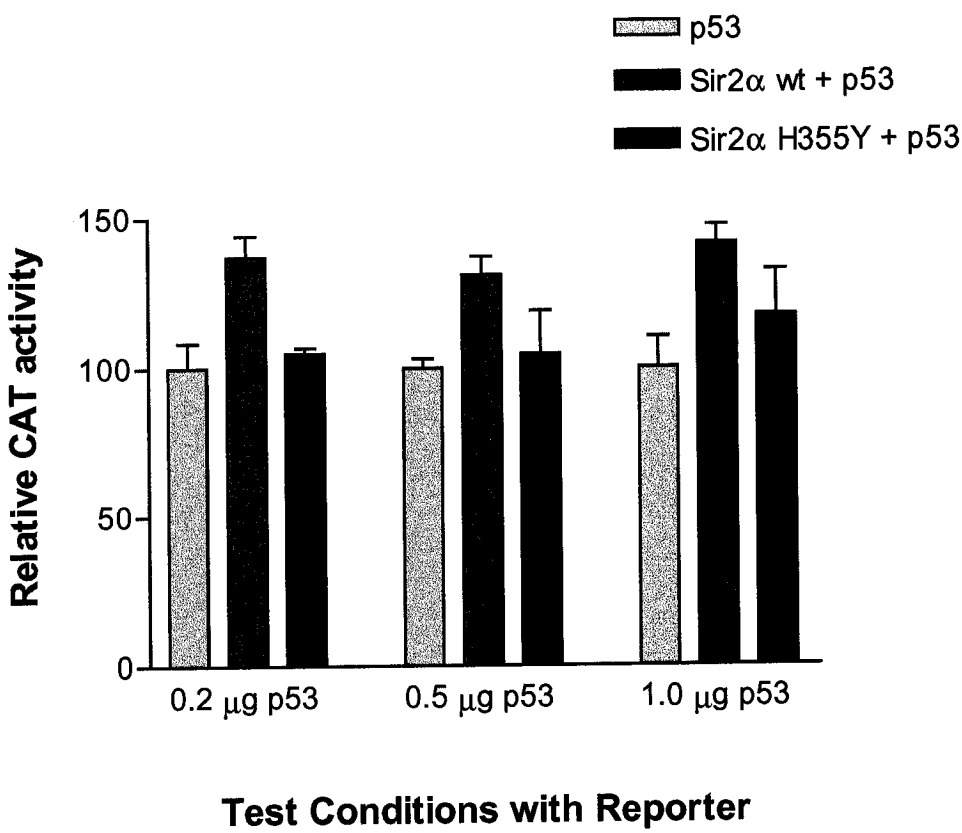

His-Ump
His-Sir2wt
His-Sir2mut
300
200
100
0
-100
Slr2-like activity

Surprisingly, the Flag-Ump-His also had histone deacetylase activity, which could be due to other proteins that are seen in the purified lysate by amido black staining (Figure 10A). This indicates that the His-Sir2α wt construct encodes an active protein that has Sir2α dependent deacetylase activity.

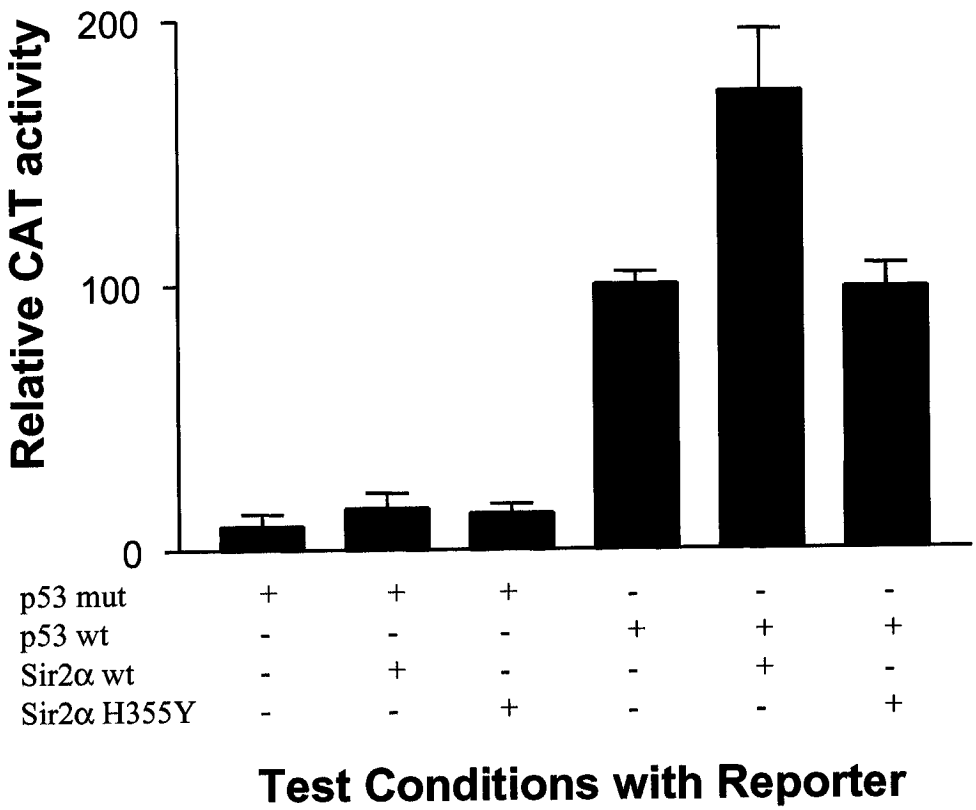
Our previous work with our Sir2α knockout embryonic stem (ES) cells showed that our Sir2α null and wildtype ES cells are not radiation sensitive and do not undergo p53-mediated arrest in G1 phase following ionizing irradiation [42]. This was not consistent with the idea that p53 is regulated by Sir2α activity, which was claimed in three papers [61-63]. We set out to reproduce these results by use of a p53 responsive-chloramphenicol acetyltransferase (CAT) reporter system. This reporter contained a maspin regulatory element upstream of a minimal TATA box promoter driving CAT expression (Figure 11B). Maspin is a serine protease inhibitor and its gene is positively regulated by p53 [64]. p53 binds directly to the p53 consensus binding site present in the maspin regulatory region. O41 cells are fibroblast cells from a patient with a p53 mutation in one allele. Expression of the other p53 allele was spontaneously lost in culture [65]. These cells were used with this reporter system because it would provide a p53 null system, giving low background readings with the reporter alone and prevent saturation of the system with endogenous p53. O41 cells were cotransfected with the CAT reporter construct, p53 alone or along with His-Sir2α wt or His-Sir2α H355Y. Referring to Figure 11A, results show that His-Sir2α wt increased CAT reporter activity, while His-Sir2α H355Y did not, as compared to p53 alone. This reporter was tested over a range concentrations of p53, which have all given the same result. In addition to this, the reporter gives negligible readout with His-Sir2α wt or His-Sir2α H355Y alone

Figure 11: The effect of the His-Sir2α wt and His-Sir2α H355Y proteins on the p53 mediated transcription from the Maspin-CAT reporter. (A) 293T cells were cotransfected with the indicated plasmids along with the Maspin-CAT reporter. Cells were harvested at 48 hrs post transfection and the CAT assay was carried out. Results were normalized to GFP expression levels. Error bars are ± S.E. (B) The CAT assay from A) was repeated with 0.2μg of p53wt or p53mut, showing negligible effect of His-Sir2α wt or His-Sir2α H355Y proteins with the reporter alone. Error bars are ± S.E. (C) Schematic of the Maspin-CAT reporter. A basal promotor (TATA box) drives CAT expression. A p53 responsive element from the Maspin upstream cDNA sequence is located upstream from the promotor and is the main consensus site in Maspin for p53 binding.

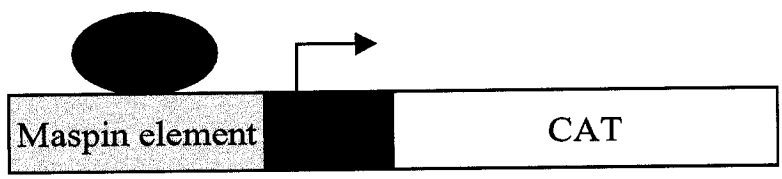
A


p53
Sir2α wt + p53
Sir2α H355Y + p53
Relative CAT activity
150
100
50
0
0.2 μg p53
0.5 μg p53
1.0 μg p53
Test Conditions with Reporter

B


Relative CAT activity
200
100
0
p53 mut + + + - - -
p53 wt - - - + + +
Sir2α wt - + - - + -
Sir2α H355Y - - + - - +
Test Conditions with Reporter

C


Maspin element
CAT

(Figure 11B). This result is the opposite to what has been previously seen by the three groups [61-63], and along with our previous work with the Sir2α null cells [42], it questions the relationship between p53 and Sir2α.

3.3 Interactions between Sir2α and MyoD

To investigate the hypothesis that Sir2α inactivates and deacetylates MyoD, a MyoD dependent luciferase reporter system was used. The 4RE-luciferase reporter contains four E box sites upstream of an SV40 promotor that drives luciferase transcription (Figure 12B). These E box sites are MyoD binding consensus sites and MyoD binding enhances luciferase activity. 293T cells were cotranfected with MyoD and Sir2α wt or Sir2α H355Y and the reporter construct. Referring to Figure 12A, results show that both Sir2α wt and Sir2α H355Y enhance MyoD activity compared to MyoD alone, which is seen by the increased luciferase reporter activity. The reporter alone, or with Sir2α wt or Sir2α H355Y, had negligible activity. This indicates that MyoD transactivational ability is not affected by Sir2α deacetylation.

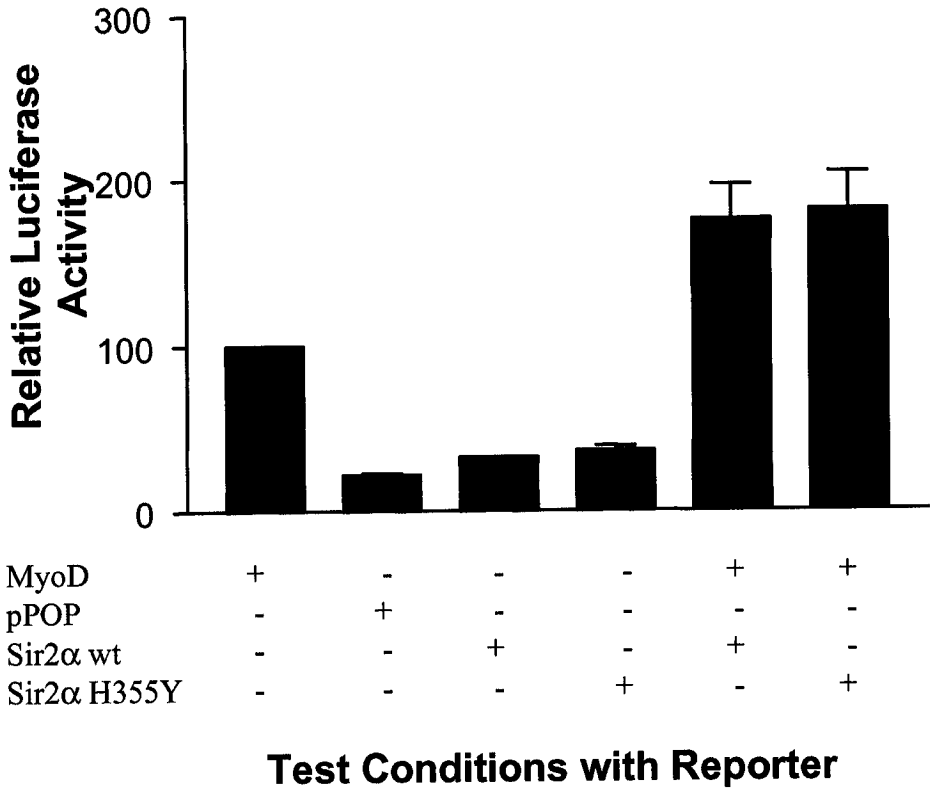
3.4 C2C12 cells- A Model for Myogenesis

3.4.1 Characterization of C2C12 cell Differentiation

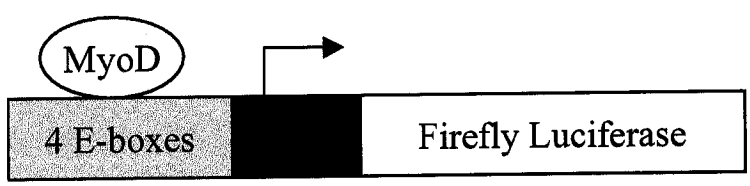
C2C12 cells are an immortalized mouse myoblast cell line that can be induced to differentiate in culture under reduced serum conditions to form multinucleated myotubes.

Figure 12: The Effect of the Sir2α wt and Sir2α H355Y proteins on the MyoD mediated transcription of the 4RE-luciferase reporter. (A) 293T cells were cotransfected with the indicated plasmids along with the 4RE-luciferase reporter. Cells were harvested at 24 hrs post transfection and the luciferase assay was carried out. No difference in effect on MyoD activity could be detected between the Sir2α wt and Sir2α H355Y. Results are based on 2 independent experiments carried out in duplicate and were normalized to protein levels. Error bars are ± S.E. (B) Schematic of the 4RE-luciferase reporter. A SV40 promotor drives luciferase expression. Four E box elements upstream of the promotor are consensus sites for MyoD binding.

A


300
200
100
0
Relative Luciferase Activity
MyoD + - - - + +
pPOP - + - - - -
Sir2α wt - - + - + -
Sir2α H355Y - - - + - +
Test Conditions with Reporter

B


MyoD
4 E-boxes
Firefly Luciferase

These cells provide an efficient model to study myogenesis and the process of myoblast differentiation. C2C12 cells were initially characterized for their expression of key myogenic factors such as myosin heavy chain (MHC) and desmin. C2C12 cells were differentiated over a 120 hrs time period, fixed and stained for MHC and desmin. Both MHC and desmin protein expression could be seen at 48 hrs and levels increased with greater differentiation of the cells (Figure 13). At 120 hrs, clear cytoplasmic staining of MHC and desmin all along the myotubes could be seen.

C2C12 cells have low levels of endogenous Sir2α protein. Upon differentiation, immunofluorescence work shows that Sir2α is initially nuclear but becomes partially cytoplasmic in the highly differentiated myotubes, as observed by an increased cytoplasmic staining (Figure 14). However, visual estimation of the western blot reveals that total levels of Sir2α actually is higher at 12 to 24 hrs and then gradually decreases over the 120 hrs total time period (Figure 15).

3.4.2 Stable Over-expression of Sir2α wt and Sir2α H355Y in the C2C12 cells

In order to test the idea that the Sir2α deacetylase activity may inhibit the overall process of myogenesis, Sir2α wt and the catalytically inactive Sir2α H355Y were overexpressed in C2C12 myoblast cells. A version of MA4 and MA5 constructs was created containing the puromycin resistance gene upstream of the Sir2α cDNA sequence. The puromycin selection marker was driven by its own pgk promotor. These

Figure 13: Cellular localization of endogenous Myosin-heavy chain (MHC) and Desmin in differentiated C2C12 cells. C2C12 cells were plated on gelatin coated coverslips and differentiated under reduced serum conditions for a period up to 120 hrs. Cells were fixed at 48 and 120 hrs, as shown in the upper and lower two panels respectively. MHC was detected by the mouse MF20 monoclonal antibody, while desmin was detected by the mouse desmin monoclonal antibody. Clear cytoplasmic staining of MHC and desmin was detected along the multinucleated myotubes.

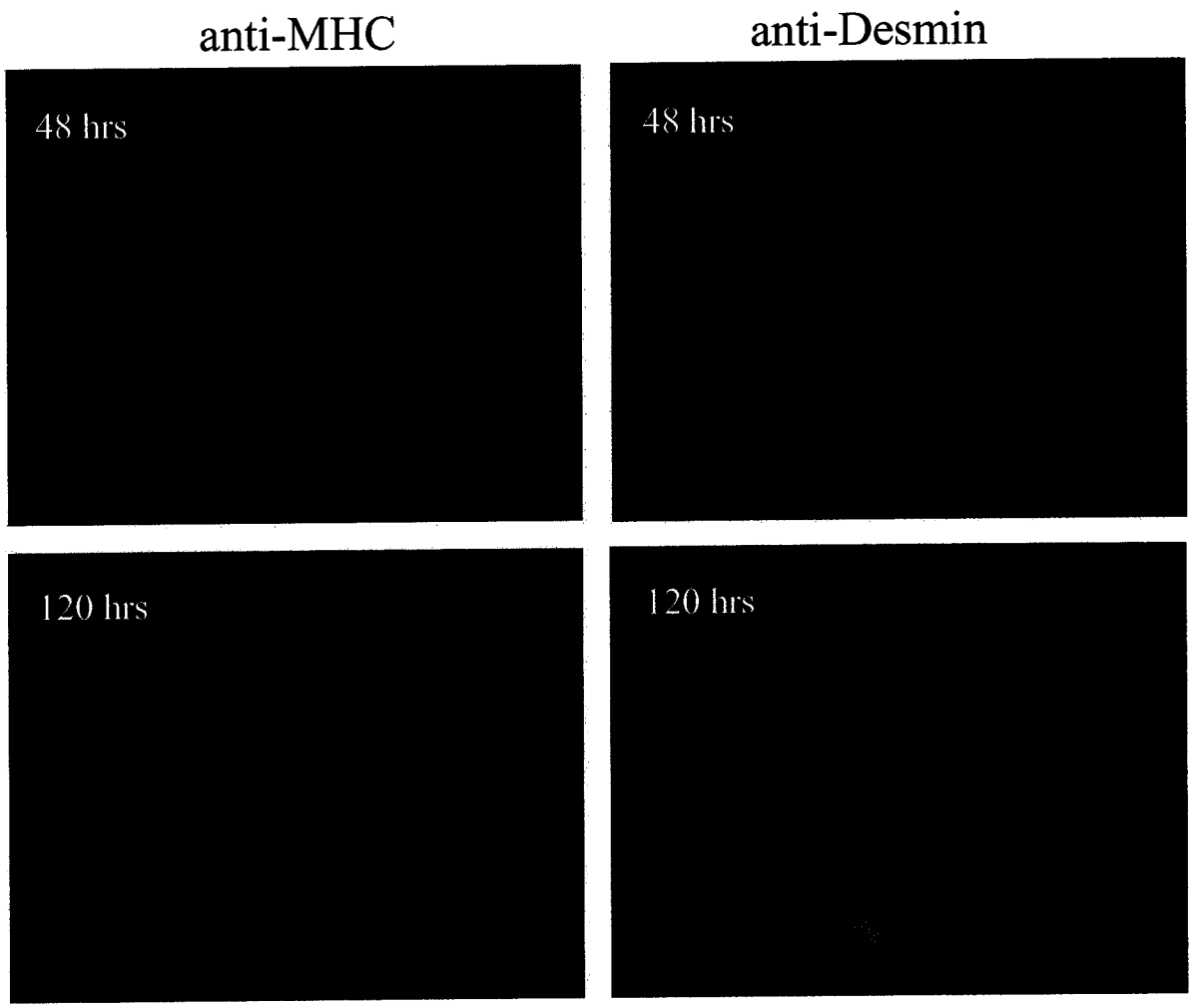

anti-MHC
anti-Desmin
48 hrs
48 hrs
120 hrs
120 hrs

Figure 14: Cellular localization of endogenous Sir2α protein during the differentiation of C2C12 cells. C2C12 cells were plated on gelatin coated coverslips and differentiated under reduced serum conditions for a period up to 144 hrs. Cells were fixed at 0, 72, 120 and 144 hrs. Sir2α was detected by the rabbit Sir2α polyclonal antibody. Sir2α staining became partially cytoplasmic by 120 hrs.

anti-Sir2α

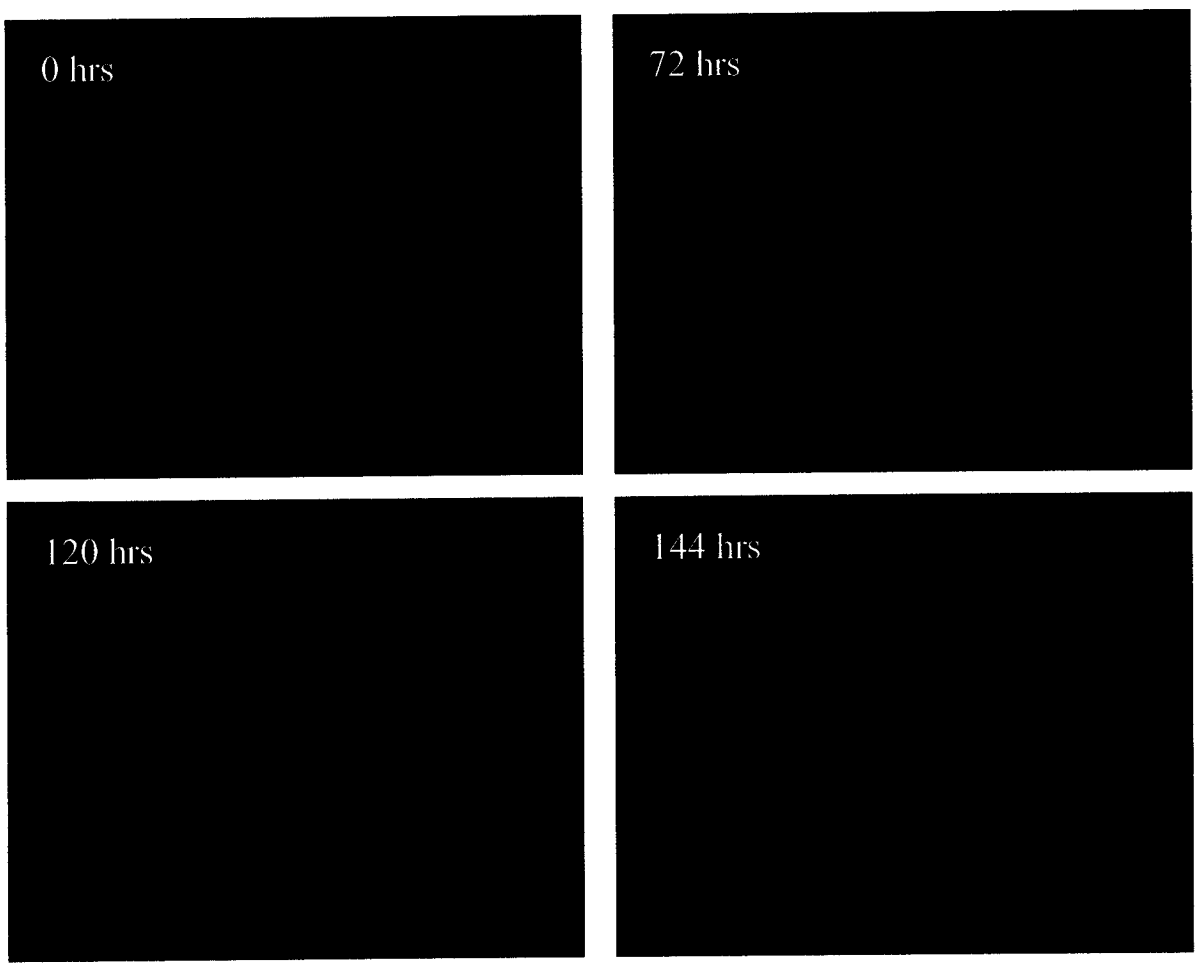
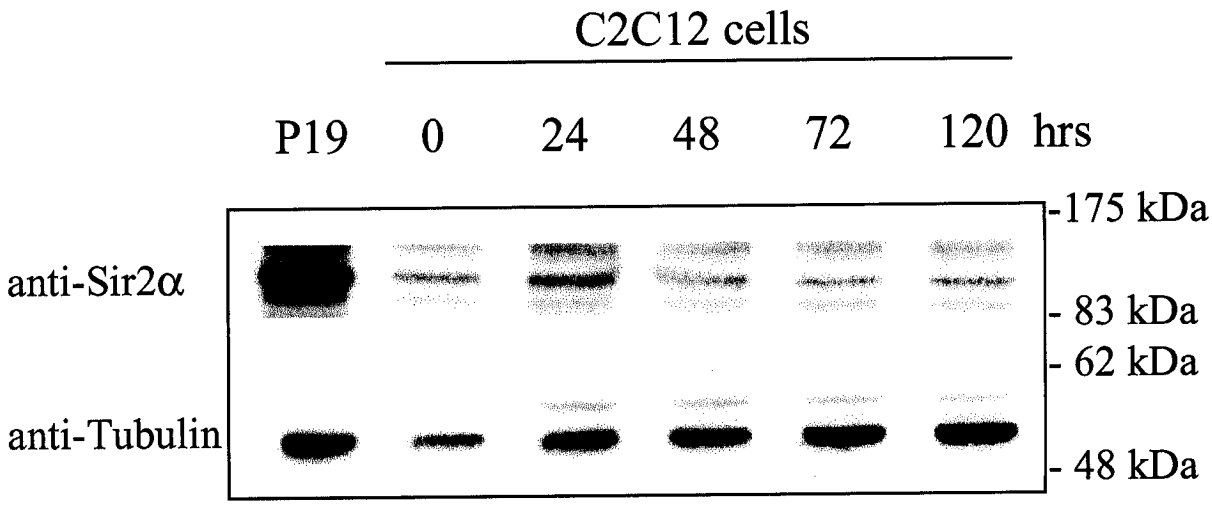


Figure 15: Endogenous Sir2α protein levels during the differentiation of C2C12 cells. C2C12 cells were differentiated under reduced serum conditions for a period up to 120 hrs. Cells were frozen at the indicated time points and protein was collected and analyzed by western blotting. Sir2α protein was detected by the rabbit Sir2α polyclonal antibody while tubulin was detected by the E7 hybridoma. Visual estimation showed that Sir2α protein levels were higher between 12 to 24 hrs and then gradually decreased.

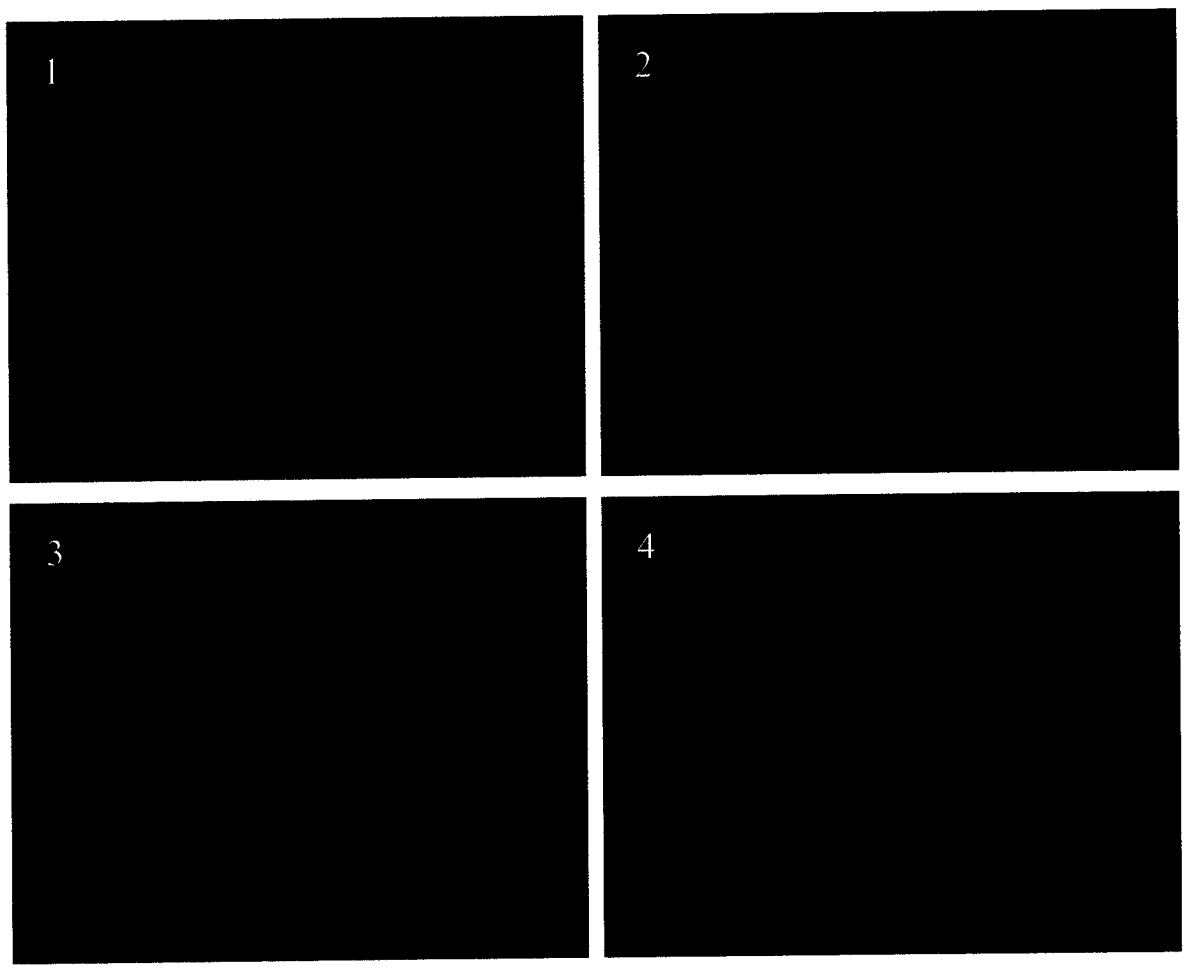

C2C12 cells
P19
0
24
48
72
120 hrs
anti-Sir2α
anti-Tubulin
-175 kDa
- 83 kDa
- 62 kDa
- 48 kDa

puroSir2α wt and puroSir2α H355Y constructs were referred to as ML19E and ML20E respectively. C2C12 cells were electroporated with puroSir2α wt, puroSir2α H355Y or a purolacZ control (KJ35), and selected for puromycin resistance for a period up to Day 12. Individual colonies were picked, grown up and then differentiated under reduced serum conditions. With the puroSir2α wt, puroSir2α H355Y or a purolacZ control tranfected clones, the clones were either highly or poorly differentiated. This is characteristic of C2C12 cells, where upon stable transformation of these cells, some clones will not differentiated for reasons unknown. We screened both the highly and poorly differentiated clones for Sir2α and Desmin protein expression by immunofluorescence.

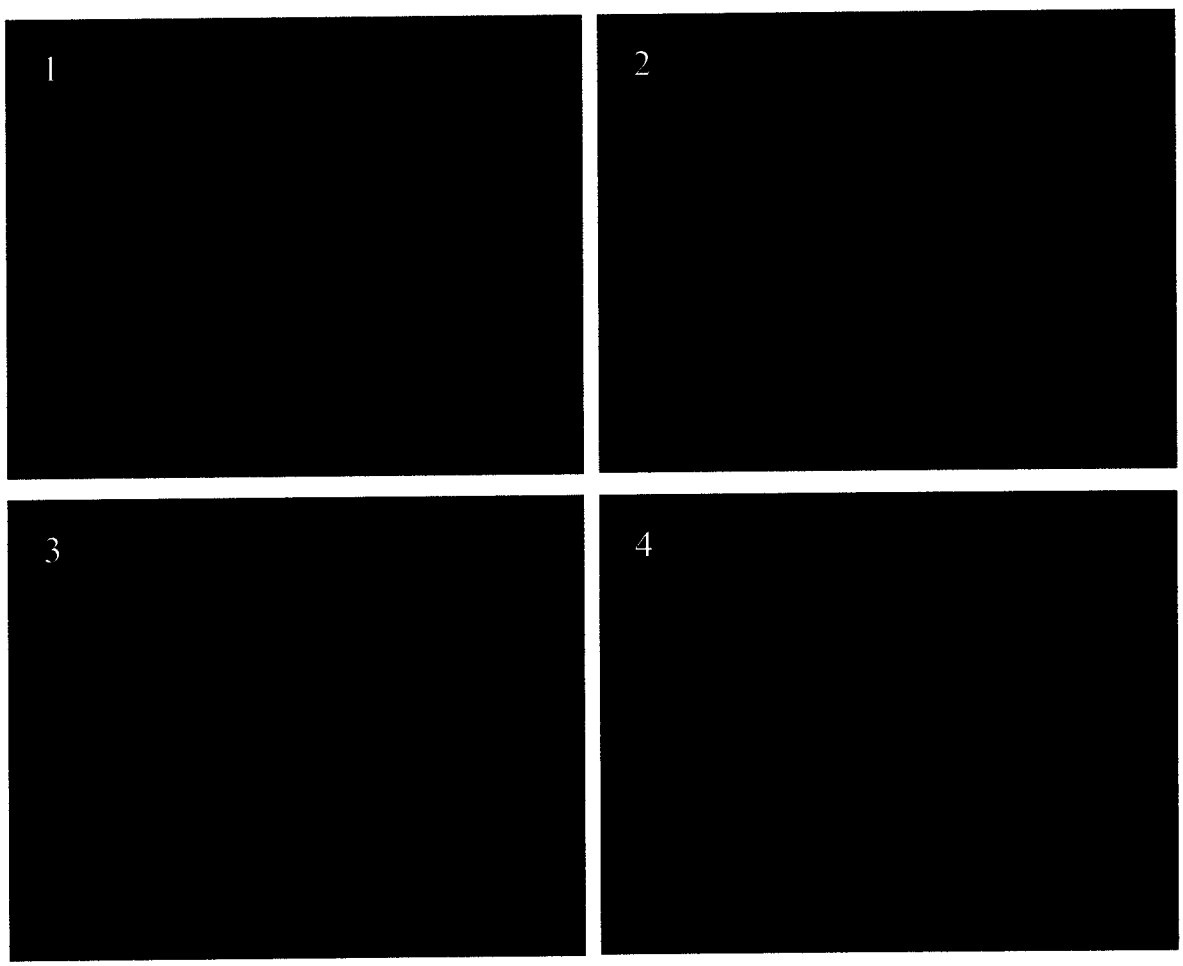
We predicted that overexpression of Sir2α wt in these C2C12 myoblast cells would result in inhibition of differentiation to myotubes, while the Sir2α H355Y would accelerate the process of differentiation. Referring to Figure 16A, in the poorly differentiated puroLacZ clones, minimal staining of Sir2α and Desmin was observed (panel 2 and 4, respectively), and the same result was seen for Sir2α wt and Sir2α H355Y clones. In the case of the highly differentiated puroLacZ clones, the clones (Figure 16A) all followed the C2C12 wildtype cell differentiation pattern. The differentiated clones were highly multinucleated myotubes, with elongated myotube shape. The desmin staining was cytoplasmic (Figure 16A, panel 1) while Sir2α staining was predominantly nuclear with some cytoplasmic staining (Figure 16A, panel 3). The same pattern of Sir2α and Desmin expression was seen with the highly differentiated puroSir2α wt and puroSir2α H355Y clones (Figure 16B), but there was some difference in the shape of the myotubes. In case of both the puroSir2α wt and puroSir2α H355Y

Figure 16: Desmin and Sir2α double staining after C2C12 cell differentiation in cells stably over-expressing protein from the puroSir2α wt and puroSir2α H355Y mammalian expression vectors. C2C12 cells were electroporated with puroLacZ, puroSir2α wt or puroSir2α H355Y expression vectors and stable colonies were picked. Cells were plated on gelatin coated coverslips and differentiated under reduced serum conditions. Each panel shows stable clones from each of the different constructs electroporated. In Panel A) Well differentiated (pictures 1 and 3) and poorly differentiated clones (pictures 2 and 4) of the lacZ control are compared. In Panel B) Well differentiated clones of Sir2α wt (pictures 1 and 3) and Sir2α H355Y (pictures 2 and 4) are compared. Desmin staining was observed by the FITC-conjugated anti-mouse IgG secondary antibody (green), while Sir2α staining was observed by the Cy3-conjugated anti-rabbit IgG secondary antibody (red).

A



B



clones, the differentiated myotubes were not as elongated in shape as compared to the puroLacZ clones and in some cases were cuboid in shape. Although there is some difference in shape between the puroLacZ control myotubes when compared to the puroSir2α wt and puroSir2α H355Y clones, the overall alterations of Sir2α levels does not affect myogenesis.

The Sir2α staining in the cells overexpressing puroSir2α wt and puroSir2α H355Y was slightly higher than the puroLacZ control. This indicates that our constructs are not abundantly being overexpressed. Electroporation theoretically inserts only one copy of the test construct into each cell, opposed to transfection techniques which can insert multiply copies of the test construct into the cell's genome. Therefore overexpression by transfection would yield a more abundantly expressed protein. Overexpression of Sir2α wt and Sir2α H355Y by transfection has been attempted in the past, but could not yield stable transformants overexpressing the Sir2α proteins. It was suspected that stable overexpression of these proteins was lethal to the cell, and only electroporations could achieve stable transformants (Appendix B). This limits our overexpression work to transient assays, where overexpression of the Sir2α wt and Sir2α H355Y is easily achieved. But the C2C12 cell differentiation process occurs over several days, and transiently expressed constructs can easily be lost during this time period.

3.4.3 Deacetylase Inhibitors

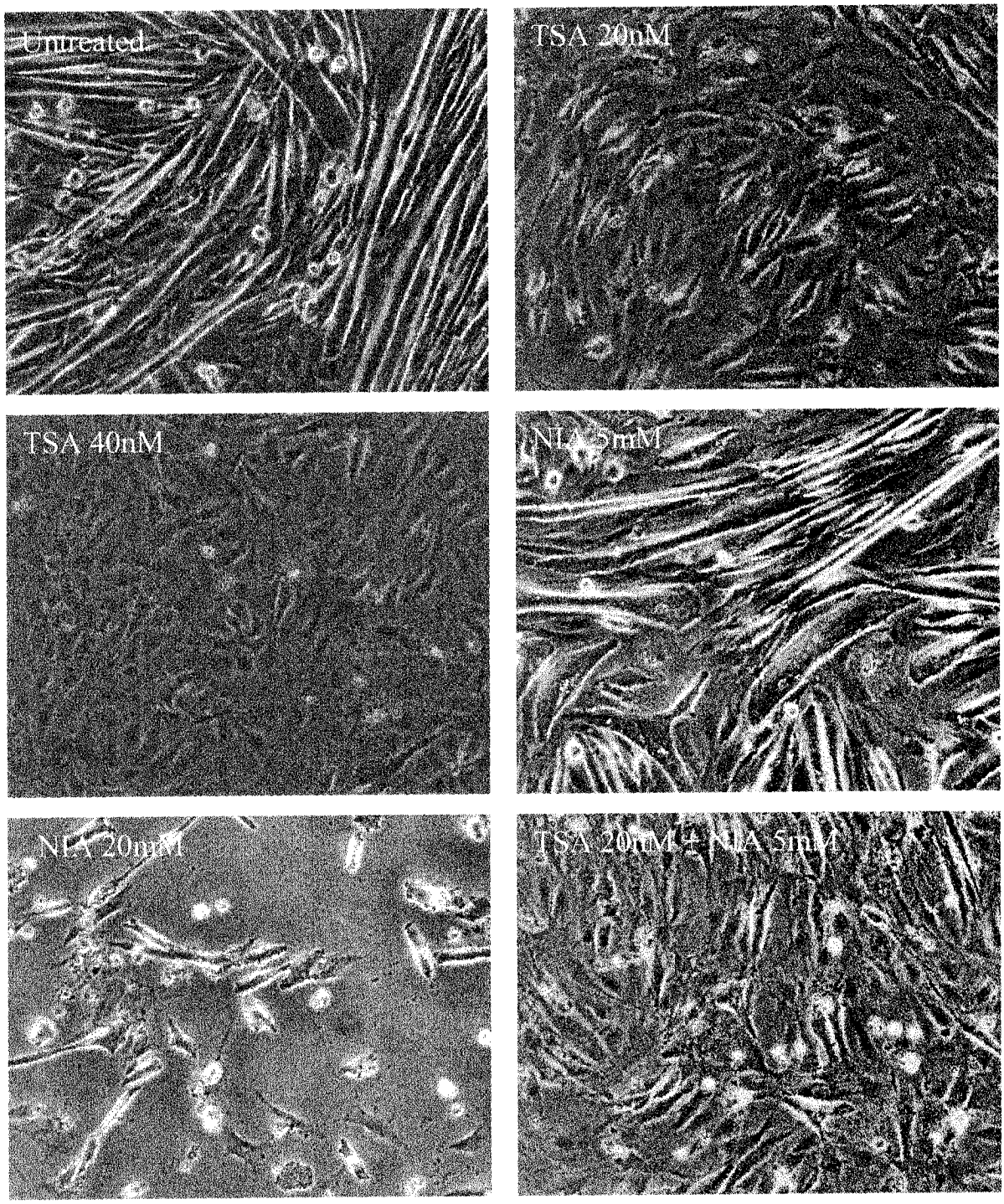
Another attempt to study Sir2α function in C2C12 cells came from our work with specific deacetylase inhibitors. The use of a Sir2α inhibitor over the process of

differentiation could easily be done, without the problems associated with the overexpression experiments. Although Sir2α does not function to inhibit MyoD, it could function to inhibit other factors involved in myogenesis. Based on our hypothesis, it was predicted that inhibition of Sir2α would result in a greater extent of differentiation in the C2C12 cells. We differentiated C2C12 cells by reduction of serum condition in the presence of TSA, nicotinamide, or both in varying concentrations. TSA is a specific Class I and II HDAC inhibitor, while nicotinamide is a Sir2α inhibitor. Referring to Figure 17, results show that TSA prevented formation of myotubes. TSA was used at a concentration of 20 nM, which has previously been used in our laboratory with other cell types. Higher concentrations of TSA was also tested with the C2C12 cells, but caused major cell death. Nicotinamide treated C2C12 cells behaved as the untreated cells, showing high levels of differentiated myotubes. Nicotinamide was tested at concentrations of 5 mM and 20 mM. Nicotinamide at a concentration of 5 mM has been shown by a number of groups to inhibit Sir2α deacetylase activity [16, 56, 62, 66]. We also tested the higher concentration, which caused cell death. The treatment of C2C12 cells with the combination of TSA and nicotinamide gave the same results as TSA alone.

Opposite to thinking, Class I and II HDAC inhibitors are known to inhibit myogenesis. Although HDACs function to inhibit myogenic factors, certain cellular cues aid in HDAC-mediated inhibition of cell cycle progression factors such as E2F. HDAC1 is known to bind to hypophosphorylated Rb and inhibit E2F. Inhibition of HDACs by TSA prevents exit from the cell cycle and thus prevents myogenesis [55], which is exactly the result we saw. Another possibility was that Sir2α may function in myogenesis in cell cycle progression pathways, similar to the Class I and II HDACs.

Nicotinamide would have inhibited myogenesis, giving us results similar to TSA. However nicotinamide treated C2C12 cells behaved as the untreated, indicating that Sir2α does not function to inhibit myogenesis and does not function in similar pathways as Class I and II HDACs in the process of myogenesis.

Figure 17: The effect of HDAC and Sir2α inhibitors on C2C12 cell differentiation. C2C12 cells were differentiated under reduced serum conditions for a period up to 120 hrs in the presence of inhibitors. Trichostatin A (TSA) and/or nicotinamide (NIA) were added at the indicated concentrations at the time of serum reduction. The inhibitor was replenished along with the media every 48 hrs. TSA is a potent inhibitor of myogenesis while NIA alone, or in combination with TSA, had no effect on the progression of myogenesis.


Untreated
TSA 20nM
TSA 40nM
NIA 5mM
NIA 20mM
TSA 20nM + NIA 5mM

Chapter 4: Discussion

We set out to find a role for Sir2α in the mammalian system. Although the Sir2α protein is highly conserved from yeast to mammals, its role in the mammalian system is unclear. Transcription factors that require acetylation for their function are potential targets for deacetylation by Sir2α. One known example is BCL-6, whose deacetylation can be catalyzed by either Sir2α or HDACs [56].

In choosing a possible target for Sir2α deacetylase activity, we looked at previous work done in our laboratory with P19 cells. This work demonstrated that P19 cells transfected with MyoD did not differentiate into muscle, while fibroblasts transfected with MyoD quickly differentiate into myotubes. P19 cells highly express Sir2α, while fibroblasts have low levels of Sir2α. Because of this differential expression of Sir2α between the cell types, it was thought that high Sir2α levels in the P19 cells may be preventing them from undergoing myogenesis. Myogenesis involves a number of HDACs that inhibit activity of key transcription factors through deacetylation of chromatin structure and transcription factors. MyoD acetylation is essential for its myogenic activity, but its deacetylation is not clearly understood. These observations taken together made myogenesis and MyoD potential targets for Sir2α deacetylase activity.

In order to determine that our Sir2α wt and Sir2α H355Y vectors expressed active and inactive protein, respectively, purified extracts of the His-Sir2α wt and His-Sir2α H355Y proteins were used in deacetylation assays. Work showed that the release of [^{3}H]-acetyl-product from the [^{3}H]-acetyl-H4-peptide substrate was inhibited by

nicotinamide only in the His-Sir2α wt purified protein sample. The His-Sir2α H355Y protein did not possess nicotinamide inhibitable activity, where the histone deacetylase activity increased with the addition of nicotinamide. This showed that the His-Sir2α wt protein possessed Sir2-like activity, and indicated that our Sir2α wt and Sir2α H355Y expression vectors encoded enzymatically active and inactive protein, respectively.

We set out to perform p53-dependent reporter assays to determine the relationship between Sir2α and p53. The maspin-CAT reporter was a p53-dependent system, where a high activity of CAT was seen with the addition of p53. Along with p53, the addition of His-Sir2α wt increased p53 activity when compared to p53 alone. However these results were contrasting to what was previously published, where three groups have shown that Sir2α wt inhibits p53-mediated transactivation of a p53-dependent luciferase reporter, while the Sir2α H355Y transfected sample was no different from p53 alone [61-63]. These reports use large amounts of Sir2α protein compared to p53, which does not reflect endogenous levels of these proteins in the cell. Work in our laboratory suggests that Sir2α levels have no effect on p53. Irradiation of wildtype and Sir2α null ES cells resulted in p53 protein elevation to the same extent in both wt and Sir2α null cells [42]. Both wildtype and Sir2α null ES cells are not radiation sensitive and do not undergo p53-mediated arrest in G1 phase following ionizing irradiation. This questions the effect of endogenous Sir2α on p53 activity.

The work with Sir2α and MyoD show that Sir2α wt and Sir2α H355Y both enhance the MyoD mediated activation of the 4RE-luciferase reporter. This indicates that the enhanced luciferase is not due to Sir2α enzymatic activity and may be caused by non-specific binding of Sir2α protein to the reporter, MyoD, or to an inhibitor of MyoD.

Overexpression of Sir2α wt or Sir2α H355Y in the C2C12 cells did not significantly alter the phenotype of the differentiated myotubes and did not alter the overall extent of differentiation. Sir2α localization was changed during the myoblast differentiation process, where a highly differentiated myotube had both nuclear and cytoplasmic staining for Sir2α. TSA treatment of C2C12 cells during the differentiation process resulted in the inhibition of myogenesis, while the Sir2α inhibitor, nicotinamide, had no effect on the myogenic process. These results demonstrate that Sir2α deacetylase activity was not involved in inhibition of MyoD transactivational ability and has no effect on myogenesis.

It is unclear whether the change in Sir2α localization in myogenesis is real or an artifact. It is known that Class II HDACs are exported out of the nucleus at the onset of myogenesis and this process is critical for myogenesis to occur. HDAC 4 and 5 export process involves a conserved NES located at the extreme carboxy termini that is recognized by the CRM1 exportin protein. There are 6 consensus calcium/calmodulin-dependent protein kinase (CaMK) phosphorylation sites in HDAC 4 and 5, out of which Ser259 and Ser498 are required for export [24, 25]. These phosphorylated serine residues create a docking site for the intracellular chaperone protein 14-3-3. It is theorized that binding of the chaperone protein unmasks the C-terminal NES sequence, which is then recognized by CRM1 and HDAC 4 and 5 are exported out of the nucleus. HDAC 4 and 5 export liberates MEF2 and allows for activation of MyoD mediated myogenesis. In the case of Sir2α, it does not have a conserved NES sequence, making it highly unlikely that it is transported out of the nucleus.

The main conclusions that we can draw from our experiments are that Sir2α does not play a role in myogenesis of C2C12 cells, and MyoD activity is not down-regulated

by Sir2α deacetylation. This indicates that Class I and II HDACs have a more important role than Sir2α in regulating MyoD activity and myogenesis. In terms of an interaction between Sir2α and p53, our experiments with the p53-responsive CAT reporter and past experiments with the Sir2α null ES cells suggest that p53 is not down-regulated by Sir2α deacetylase activity. The role of Sir2α in the mammalian system remains to be determined.

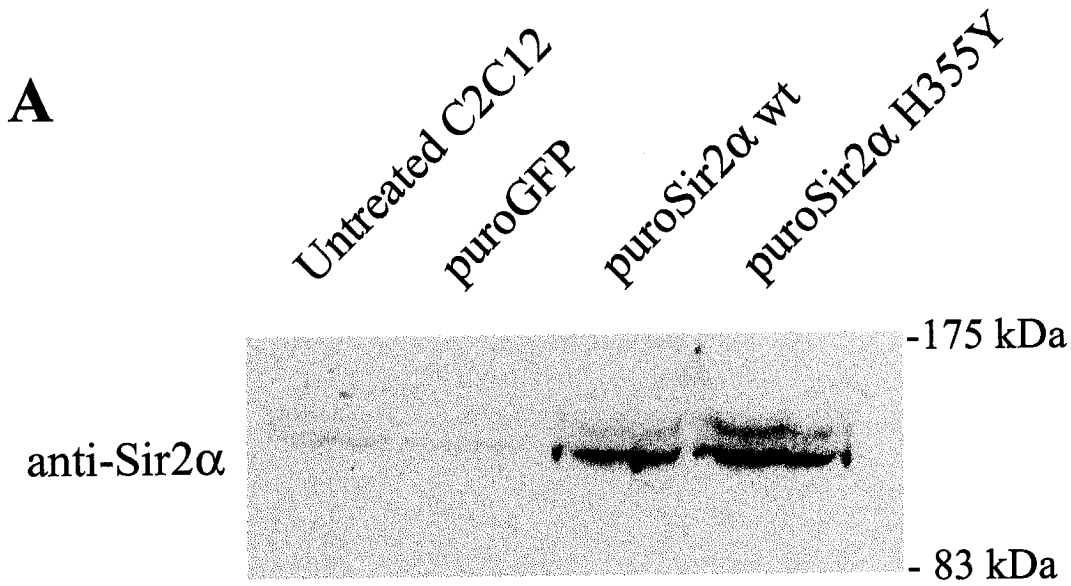
Appendix A: List of Plasmids

Plasmid	Description and origin of plasmid
Sir2α wt (MA4)	Pgk promoter driving full-length Sir2α wt expression. Generated in collaboration with Chris Kamel.
Sir2α H355Y (MA5)	Pgk promoter driving full-length Sir2α H355Y expression. Generated in collaboration with Chris Kamel.
His-Sir2α wt (CK1)	Pgk promoter driving RGS-His_6 tagged Sir2α wt expression. Generated by Chris Kamel.
His-Sir2α H355Y (CK2)	Pgk promoter driving RGS-His_6 tagged Sir2α H355Y expression. Generated in collaboration with Chris Kamel.
puroLacZ (KJ35)	Pgk promoter driving puromycin gene expression upstream of the lacZ gene. Generated by Karen Jardine.
puroSir2α wt (ML19E)	Pgk promoter driving puromycin gene upstream of pgk-Sir2α wt (MA4). Generated by Madeleine Lemieux.
puroSir2α H355Y (ML20E)	Pgk promoter driving puromycin gene upstream of pgk-Sir2α H355Y (MA5). Generated by Madeleine Lemieux.
pPOP	Mammalian expression vector containing the pgk 5' and 3' sequences with a multiple cloning site. Generated by Karen Jardine.
Puromycin	Pgk promoter driving puromycin gene expression. Generated by Peter W. Laird.
MyoD (CA1)	Pgk promoter driving MyoD gene expression. Generated by Christine Armour.
GFP (KJ162)	Pgk promoter driving EGFP expression. EGFP encodes the GFPmut1 varient which has been optimized for brighter fluorescence and higher expression in mammalian cells. Generated by Karen Jardine.
puroGFP (ML8)	Pgk promoter driving puromycin gene upstream of pgk-GFP (KJ162). Generated by Madeleine Lemieux.

p53	CMV promoter driving p53 wt expression. Obtained from Dr. Bruce McKay, Ottawa, ON.
Maspin-CAT	p53 response element from the maspin gene located upstream of a TATA box, driving CAT expression. Obtained from Dr. Bruce McKay, Ottawa, ON.
Flag-Ump-His	N-terminal Flag tagged and C-terminal RGS-His_6 tagged Ump expression driven by the CMV promoter. Obtained from Dr. Doug Gray, Ottawa, ON.
4RE-luciferase	4 E box elements upstream of the SV40 promoter driving luciferase expression. Obtained from Dr. Mike Rudnicki, Ottawa, ON.

Appendix B: Other Results

Differences in Sir2α wt and Sir2α H355Y protein expression in C2C12 cell after stable overexpression by electroporation or tranfection. (A) C2C12 cells were electroporated with puroGFP, puroSir2α wt or puroSir2α H355Y. (B) C2C12 cells were transfected with GFP, His-Sir2α wt or His-Sir2α H355Y along with the puromycin resistance vector. In both cases, cells were placed under puromycin selection 48 hrs after electroporation or tranfection. Stable colonies were pooled and analyzed by western blotting. Sir2α protein was detected by the rabbit Sir2α polyclonal antibody in (A) and with the His commercial antibody in (B). Only the electroporated cells overexpress the Sir2α proteins, at 120 kDa.


A
Untreated C2C12
puroGFP
puroSir2α wt
puroSir2α H355Y
-175 kDa
anti-Sir2α
- 83 kDa
B
Untreated C2C12
GFP
His-Sir2α wt
His-Sir2α H355Y
-175 kDa
anti-His
- 83 kDa

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